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GLYCAN-BASED INTERACTIONS OF STREPTOCOCCUS PNEUMONIAE AND THE HOST

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Cover: Fluorescent microscopy picture of A549 lung epithelial cells and *Streptococcus pneumoniae* TIGR4. Sialic acids on the cell surfaces are stained with FITC-labelled *Sambucus nigra* lectin (green), nuclei are stained with DAPI (blue) and pneumococci are labelled with nile red (red).

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ABSTRACT

Streptococcus pneumoniae is commonly found as an asymptomatic colonizer of the nasopharynx of children, but it can also translocate to normally sterile body sites and cause severe diseases, like pneumonia, septicemia or meningitis. Pneumococci spread via aerosols. Upon entry into the upper respiratory tract of the host, glycoconjugates with terminal sialic acids (Sias) are among the first structures pneumococci encounter. Hence, they play an important role in pneumococcal pathogenesis. Moreover, glycans are also implicated in the recognition of microbial pathogens by the innate immune system, as many ligands of Toll-like receptors are glycoconjugates. Both aspects of glycan-based pneumococcal-host interactions were studied in this thesis.

In most mammals, the Sia *N*-acetylneuraminic acid (Neu5Ac) is converted into *N*-glycolylneuraminic acid (Neu5Gc) by the cytidine-monophosphate-*N*-acetylneuraminic acid hydroxylase (CMAH). However, humans lack Neu5Gc due to a deletion in *CMAH*, instead they overproduce Neu5Ac. We reported a faster disease progression in *Cmah*$^{-/-}$ versus wild-type (wt) mice after pneumococcal challenge and an upregulation of pneumococcal sialidase NanA and the main sialic acid transporter SatABC in response to Neu5Ac as compared with Neu5Gc, which was mediated by the response regulator CiaR.
Moreover, we detected higher pneumococcal adhesion rates to cells presenting Neu5Ac than Neu5Gc. *In vitro*, higher bacterial adherence downregulated IL-8 secretion, and *in vivo*, pneumococcal pyruvate oxidase (SpxB) and pneumolysin contributed to a reduced immune response in *Cmah*$^{-/-}$ compared with wt mice after intranasal challenge.

Influenza infections lead to changes in the pulmonary environment and sensitize for a pneumococcal infection. We observed higher protein concentrations, increased numbers of dead cells as well as upregulated hydrogen peroxide concentrations in bronchoalveolar lavages of influenza- versus mock-infected mice. The increased virus-mediated stress in the lower respiratory tract mediated an upregulation of the pneumococcal serine protease HtrA during influenza/pneumococcal coinfection. A mutant of HtrA was severely attenuated in a murine coinfection model, suggesting an important role of HtrA in pneumococcal outgrowth following primary influenza infection.

Dendritic cells link the innate with the adaptive immune system. We found an RNA-mediated recognition of pneumococci by TLR3 in dendritic cells, which induced the secretion of the cytokine IL-12. Moreover, in influenza/pneumococcal coinfections, the virus upregulated TLR3 expression, which led to an enhanced production of IL-12 by dendritic cells.

In summary, we show that glycan-mediated interactions of *S. pneumoniae* and the host play a major role in pneumococcal host tropism and strongly affect pneumococcal virulence, as well as innate immune responses.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following publications, which are referred to in the text by Roman numerals:

I. **HENTRICH K.**, LÖFLING J., PATHAK A., NIZET V., VARKI A., HENRIQUES-NORMARK B.
Streptococcus pneumoniae senses a human-like sialic acid profile via the response regulator CiaR.
Cell Host Microbe. 2016 Sep 14;20(3):307-17.

II. **HENTRICH K.**, SENDER V., PATHAK A., HENRIQUES-NORMARK B.
Human sialic profiles mediate increased pneumococcal adhesion and immune evasion.
Manuscript

III. SENDER V., **HENTRICH K.**, PATHAK A., NORMARK S., HENRIQUES-NORMARK B.
Mechanism for enhanced bacterial burden in the lower respiratory tract of mice during influenza/pneumococcal coinfection.
Manuscript

IV. SPELMINK L., SENDER V., **HENTRICH K**., KURI T., PLANT L., HENRIQUES-NORMARK B.
Toll-like receptor 3/TRIF-Dependent IL-12p70 secretion mediated by *Streptococcus pneumoniae* RNA and its priming by influenza A virus coinfection in human dendritic cells.
MBio. 2016 Mar 8;7(2):e00168-16.

Publications by the author, which are not included in the thesis

SCHULTE T., LÖFLING J., MIKAELSSON C., KIKHNEY A., **HENTRICH K**., DIAMANTE A., EBEL C., NORMARK S., SVERGUN D., HENRIQUES-NORMARK B., ACHOUR A.
The basic keratin 10-binding domain of the virulence-associated pneumococcal serine-rich protein PsrP adopts a novel MSCRAMM fold.
Open Biol. 2014 Jan 15;4:130090.

ORRSKOG S., ROUNIOJA S., SPADAFINA T., GALLOTTA M., NORMAN M., **HENTRICH K.**, FÄLKER S., YGBERG-ERIKSSON S., HASENBERG M., JOHANSSON B., UOTILA L.M., GAHMBERG C.G., BAROCCHI M., GUNZER M., NORMARK S., HENRIQUES-NORMARK B.
Pilus adhesin RrgA interacts with complement receptor 3, thereby affecting macrophage function and systemic pneumococcal disease.
MBio. 2012 Dec 26;4(1):e00535-12.

MONTEIRO C., PAPENFORT K., **HENTRICH K.**, AHMAD I., LE GUYON S., REIMANN R., GRANTCHAROVA N., RÖMLING U.
Hfq and Hfq-dependent small RNAs are major contributors to multicellular development in *Salmonella enterica* serovar Typhimurium.
RNA Biol. 2012 Apr;9(4):489-502.

CONTENTS

LIST OF ABBREVIATIONS

AcPh	Acetyl phosphate
AIM2	Absent in melanoma 2
AMs	Alveolar macrophages
AMPs	Antimicrobial peptides
AOM	Acute otitis media
ASC	Apoptosis-associated speck-like protein containing CARD
Asn	Asparagine
BAL	Bronchoalveolar lavage
CAP	Community-acquired pneumonia
CARD	Caspase recruitment domain
CD	Cluster of differentiation
CD33r Siglec	CD33-related Siglec
CFU	Colony-forming units
CMAH	CMP-Neu5Ac hydroxylase
CO_2	Carbon dioxide
CR	Complement receptor
CRAMP	Cathelicidin-related antimicrobial peptide
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
Fuc	Fucose
Gal	Galactose
GalNAc	*N*-acetylgalactosamine
GBS	Group B streptococci
Glc	Glucose
GlcNAc	*N*-acetylglucosamine
hBD	Human β-defensin
HIV	Human immunodeficiency virus
HK	Histidine kinase
HNP	Human neutrophil peptides
HtrA	High temperature requirement A
H_2O_2	Hydrogen peroxide
IAV	Influenza A virus
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPD	Invasive pneumococcal disease
IRF	Interferon regulatory factor
LPS	Lipopolysaccharide
LRT	Lower respiratory tract
LTA	Lipoteichoic acid
MAAII	*Maackia amurensis* lectin II
Man	Mannose
ManNAc	*N*-acetylmannosamine
MARCO	Macrophage receptor with collagenous structure
MHC	Major histocompatibility complex

MIP	Macrophage inflammatory protein
MOI	Multiplicity of infection
MyD88	Myeloid differentiation factor 88
NETs	Neutrophil extracellular traps
Neu5Ac	*N*-acetylneuraminic acid
Neu5Gc	*N*-glycolylneuraminic acid
NF-κb	Nuclear factor κb
NLR	NOD-like receptor
NOD	Nucleotide-binding oligomerization domain
PAMPs	Pathogen associated microbial patterns
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PCV	Pneumococcal conjugate vaccine
PGN	Peptidoglycan
Ply	Pneumolysin
PPSV	Pneumococcal polysaccharide vaccine
PRR	Pattern recognition receptor
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RR	Response regulator
Ser	Serine
Sia	Sialic acid
Siglec	Sialic acid binding Ig-like lectins
SNA	*Sambucus nigra* lectin
SP-A, SP-D	Surfactant protein-A, Surfactant protein-D
SpxB	Streptococcal pyruvate oxidase B
STING	Stimulator of interferon genes
TCS	Two-component system
T_H-cells	Helper T-cells
Thr	Threonine
TIR	Toll-interleukin 1 (IL-1) receptor
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
TRIF	TIR-domain-containing adapter protein-inducing interferon-β
WT	Wild-type

1 INTRODUCTION

1.1 *Streptococcus pneumoniae*

In 1881, George Miller Sternberg and Louis Pasteur isolated diplococci after injecting human saliva into rabbits [1, 2]. In 1886, Fraenkel used the name *Pneumococcus* for the first time, referring to the pneumonia these bacteria cause. In the same decade, Christian Gram developed a staining to visualize bacteria in patient samples. He discovered lancet-shaped gram-positive cocci in the lung specimens of patients who died of pneumonia and named them "the cocci of croupous pneumonia". In 1920, the isolate was given the name *Diplococcus pneumoniae*, due to its microscopic morphology, which was seen in the gram-stain. It took until 1974 that these bacteria were referred to as *Streptococcus pneumoniae* [3]. However, the term "*Pneumococcus*" is still frequently used.

Pneumococcal colonies appear to be small and greyish on blood agar plates after an overnight incubation at 37°C and 5% carbon dioxide (CO_2). Moreover, they are α-hemolytic, as they oxidize hemoglobin. To date, we know of at least 97 different pneumococcal serotypes, which differ in the composition of their polysaccharide capsule [4]. The presence of the capsule leads to the mucoid appearance of pneumococcal colonies on solid agar. Another characteristic of most *S. pneumoniae* isolates is the sensitivity to optochin, which allows differentiating them from *S. viridans* [5, 6] (Figure 1). However, this test is not sufficient to identify pneumococci, since there have been recent reports of optochin-resistant isolates [7].

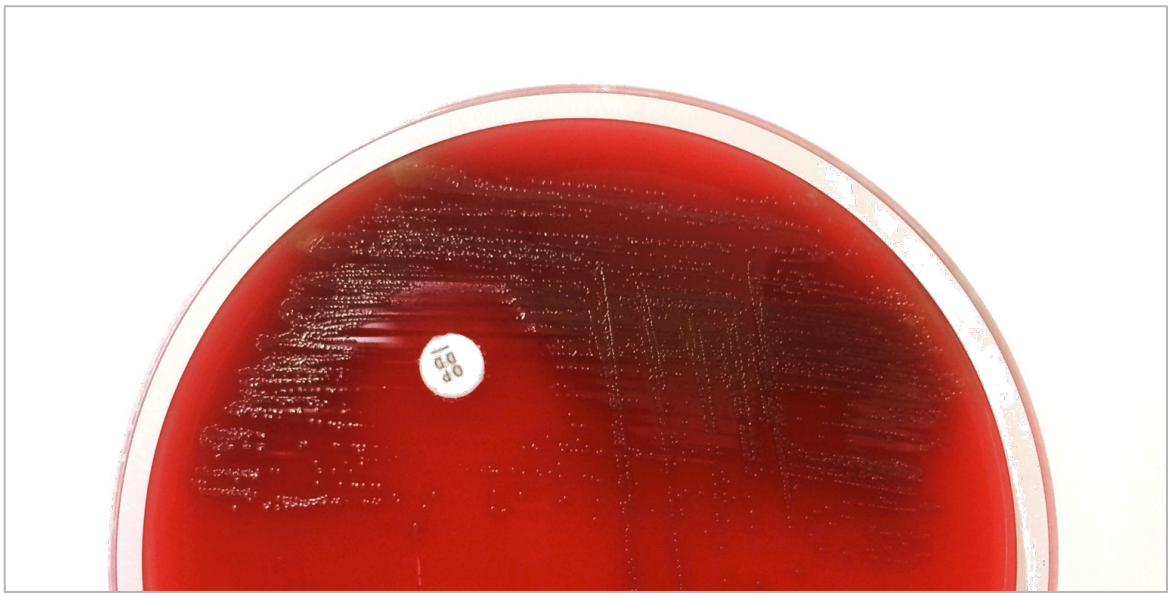

Figure 1 Optochin sensitivity of the α-hemolytic *S. pneumoniae* TIGR4. TIGR4 was streaked out on a blood agar plate and cultured at 37°C in the presence of 5% CO_2. The sensitivity to optochin was tested with a disk diffusion method.

S. pneumoniae was the first bacterium shown to be naturally competent. In 1928, Griffith showed that viable non-virulent pneumococci turned into virulent ones when they were injected into mice together with heat-killed pathogenic isolates. He hypothesized that the dead pneumococcal strain provides a certain protein that enables the attenuated bacteria to be virulent [8]. It took 16 more years to show that the exchanged substance, responsible for the increased virulence, was not protein, but deoxyribonucleic acid (DNA) [9].

1.1.1 Pneumococcal colonization and disease

Pneumococci are human-adapted and often found asymptomatically colonizing the upper respiratory tract. Nonetheless, *S. pneumoniae* can cause diseases ranging from benign respiratory tract infections, e.g. sinusitis or otitis media, to severe diseases like pneumonia, septicaemia and even meningitis [10], making pneumococcal infections a major cause of global childhood mortality, leading to about 11% of all deaths among children below the age of five [11] (Figure 2).

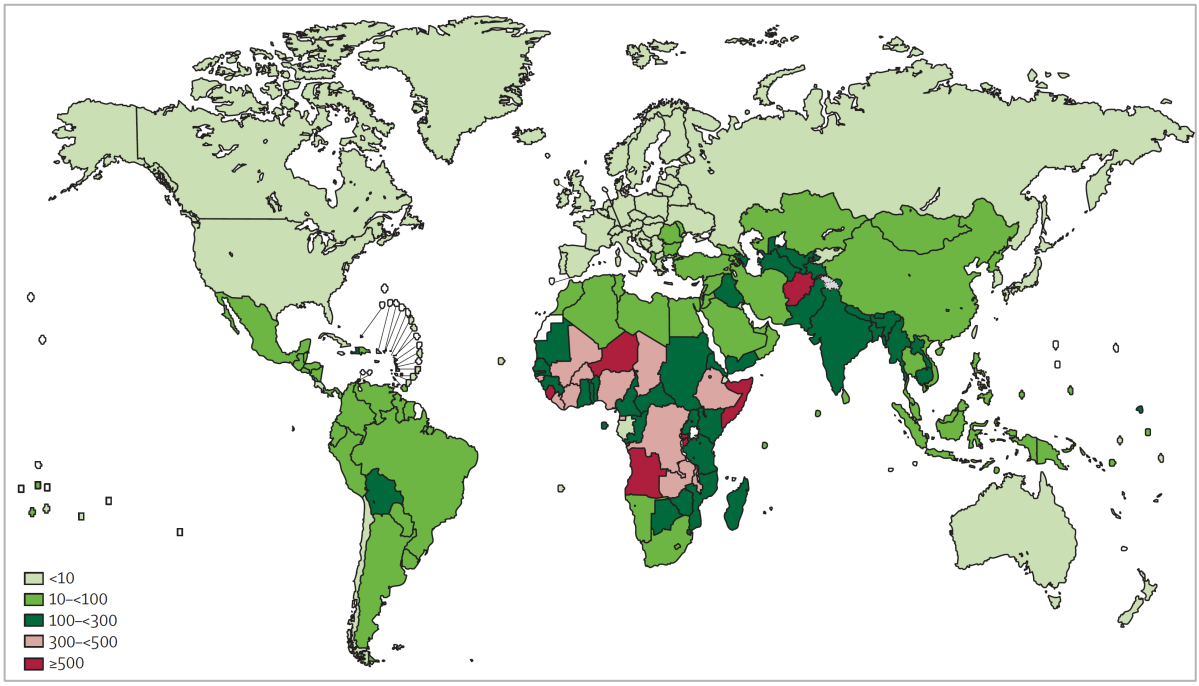

Figure 2 Pneumococcal mortalities of children younger than 5 years. The graph represents the number of deaths per 100 000 HIV-negative children caused by pneumococcal infections. The picture was adopted from [11].

Colonization

Pneumococcal colonization is believed to occur via the spread of pneumococci-containing aerosols. Before their eventual clearance by the immune system, pneumococci can colonize the upper respiratory tract for several weeks or months [12]. Especially pre-school age children and those attending day care centers are found to be the main reservoir for *S. pneumoniae* [13, 14], with carriage rates up to 60% [10, 15]. Less than 10% of adults are carriers, although a higher colonization rate was found in parents of small children [16]. Once these bacteria establish colonization, they can replicate and form biofilms and/or translocate to other sites and cause disease.

Otitis media and sinusitis

S. pneumoniae is one of the causative agents of acute otitis media (AOM), an inflammation of the middle ear, affecting up to 85% of all children at the age of 3 years [17]. In Sweden and the US, AOM represents the most common infectious disease to prescribe antibiotics to children [18]. However, recurrent or persistent AOM can lead to complications and severe sequelae, e.g. conductive hearing loss, labyrinthitis, perforation of the tympanic membrane or even meningitis [19].

Although viruses cause most of the sinusitis infections, *S. pneumoniae* is among the most common bacterial agents that can lead to infections of the paranasal sinus cavity [20]. While most viral sinusitis episodes resolve completely after 10 days, bacterial rhinosinusitis is usually more persistent [21].

Pneumonia

Pneumococcal infections of normally sterile sites of the body, like the lower respiratory tract, the blood or meninges, are considered invasive pneumococcal diseases, also called IPD [22]. An inflammation of the lungs is called pneumonia. *S. pneumoniae* is the most common cause of community-acquired pneumonia (CAP), followed by *Haemophilus influenzae*, respiratory viruses or *Mycoplasma pneumoniae* [23]. CAP represents a huge financial and clinical burden throughout the whole world [24] and accounts for 19% of all mortality cases in children below the age of 5 years. The distribution of pneumonia-caused deaths differs greatly between geographical regions and their economic situation, with 50% of all pneumonia-related mortalities occurring in the African and only 2% - 3% in the European and American region [25].

Bacteraemia and sepsis

20-30% of the children with pneumonia develop bacteraemia, the occurrence of bacteria in the blood stream [26]. The consequence can be sepsis, a life-threatening systemic infection. CAP and health-care associated pneumonia in children and adults are the pre-conditions, which lead to 50% of all sepsis cases. Especially, very young children below 1 year of age and elderly people of more than 65 years of age are prone to develop sepsis [27].

Meningitis

Since the introduction of the vaccine against *H. influenzae* type b, *S. pneumoniae* and *Neisseria meningitidis* present the major causes of bacterial meningitis in children nowadays [26, 28]. A study conducted in the US, observed that mortality rates in children were less than 10%, but almost half of the surviving patients suffered from severe sequelae, like hearing loss, brain infarcts, brain abscesses or hydrocephalus [29]. European studies describe a fatality rate of 30% in adults after pneumococcal meningitis and cognitive deficits in 26% of the survivors [30, 31]. As it is the case for pneumonia, the mortality rates after pneumococcal meningitis are significantly higher in developing than in developed countries [11].

Risk factors

As discussed before, the age determines the risk of IPD, affecting especially very young children with their naïve immune system, elderly people of an age of more than 65 years due to their weakening immune response, and individuals with co-morbidities, e.g. diabetes or different immunodeficiencies [32]. Environmental and social factors, such as the number of siblings, financial income, and smoking affect pneumococcal colonization. Furthermore, the ethnic background was also shown to determine the risk of a pneumococcal infection, with African Americans and Native Americans having a high probability of developing IPD [33].

Coinfections with viral pathogens, especially with influenza A virus (IAV, see below) and human immunodeficiency virus (HIV), have been shown to significantly promote pneumococcal infections [32, 34].

Pneumococcal coinfection with Influenza A virus

During influenza pandemics, most fatalities are not caused by the virus alone, but by a secondary infection with a bacterial pathogen [35]. In 1918, the “Spanish flu” was caused by subtype H1N1. This pandemic killed more than 50 million people, which primarily died due to superinfections with *S. pneumoniae* [36]. In 1957, IAV subtype H2N2 caused the “Asian flu”, leading to more than 1 million deaths partially due to pneumonia caused by *Staphylococcus aureus.* The severe and about 500 000 fatal cases of the “Hong Kong flu” in

1968 were mainly caused by co-infections of influenza subtype H3N2 and pneumococci [37-39]. The next influenza pandemic occurred 41 years later, and is known as the so-called "swine flu". In 2009, infections with H1N1 led to about 200 000 deaths with mainly young adults to be affected. Like former pandemics, severe cases and fatalities were mainly caused by *S. aureus* and *S. pneumoniae* [40]. Although it was first believed that the introduction of antibiotics against *S. pneumoniae* was responsible for the shift from secondary infections with pneumococci to co-infection with other pathogens, strain-related changes in the virus and/or the bacteria are more probable to be responsible for variations in the severity of influenza pandemics. Additionally, the introduction of the pneumococcal vaccine is also believed to account for lower numbers of coinfections with *S. pneumoniae* during the pandemic in 2009 [35].

1.1.2 Treatment and prevention of pneumococcal infections

Treatment

The treatment of choice of pneumococcal infections is a therapy with antibiotics. Since the introduction in the 1940s, penicillin was used against infections with *S. pneumoniae*. β-lactam-antibiotics, like penicillin, inhibit the bacterial cell-wall synthesis by interfering with penicillin-binding proteins (PBPs). Mutations in several different genes, e.g. pneumococcal PBPs or the cell-wall muropeptide branching enzyme MurM, are shown to promote resistance development to penicillins [41]. Already in the 1960s, reports about β-lactam-resistant pneumococci were published [42], and in 2013, Sweden reported that 6.8% of all IPD isolates were non-susceptible to penicillins [43].

Following the isolation of increasing numbers of penicillin-resistant pneumococci, the use of macrolides and fluoroquinolones has become more common to treat respiratory tract infections. Macrolides, like erythromycin or the semi-synthetic azithromycin, inhibit bacterial protein synthesis by binding to the 50S ribosomal subunit and inhibit protein elongation [44]. Fluoroquinolones, e.g. moxifloxacin, are synthetically produced antibiotics that bind to type II topoisomerase enzymes, like DNA gyrase, thereby preventing DNA replication and cell division. Macrolide-resistant pneumococci have been isolated in several countries with resistance rates up to 80% [45]. In 2013, 6.5% of invasive pneumococci, isolated in Sweden, were non-susceptible to macrolides [43]. A study from the same year, conducted in the US, reported that about one third of all cases of IPD were caused by pneumococci, which were resistant to several antibiotics [46].

Prevention

In order to prevent the spread of antibiotic-resistant pneumococci and the development of IPD, a pneumococcal vaccine was introduced. To date, there are 4 different pneumococcal vaccines available: A pneumococcal polysaccharide vaccine (PPSV23) and pneumococcal conjugate vaccines (PCV7, PCV10 and PCV13), which are shown to protect against the most common pneumococcal serotypes linked to IPD (Table 1).

Table 1 Pneumococcal vaccines currently available [47]

Name	Supplier	Licensed	Serotypes covered
PPSV23 Pneumovax®23	Merck	1983	1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, 33F
PCV7 Prevnar®/ Prevenar®	Pfizer	2000	4, 6B, 9V, 14, 18C, 19F, 23F
PCV10 Synflorix™	GSK	2009	1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F
PCV13 Prevenar 13®	Pfizer	2010	1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F

In 1983, a pneumococcal vaccine covering 23 different serotypes was licensed. This vaccine is polysaccharide-based and provokes an antibody response against the polysaccharide capsule of pneumococci, which promotes opsonophagocytosis. However, polysaccharide-based vaccines elicit no or only weak immune responses in very young children and adults and fail to induce immune memory due to their T-cell independent response (see chapter 1.2.2) [48].

In 2000 and 2001, the first PCV was licensed in the US and Europe, respectively [49]. PCVs consist of 7, 10 or 13 different capsular polysaccharides, which are coupled to protein carriers, a non-toxic variation of the diphtheria toxin, CRM197 (PCV7 and PCV13) or protein D from *H. influenzae* (PCV10). These vaccines cause T-cell dependent immune responses and B-cell memory [50]. The introduction of PCVs into national vaccination programmes in Europe decreased pneumococcal infections drastically in vaccinated individuals, but also in non-vaccinated people because of herd-immunity. In Germany, the introduction of PCV7 was recommended from 2006 onwards and led to a vaccine-coverage of 84% in 2007. In 2008, Germany reported a 50% reduction in IPD cases in children below the age of 2 years [51]. Sweden incorporated PCVs into the national immunization programme in 2009. Of all children born in 2013 in Sweden, 96.6% were vaccinated against pneumococcal infections by the age of 2 years [52], which led to a considerably reduction of IPD cases in vaccinated children [53].

However, although PCVs drastically reduce the nasopharyngeal carriage rates and IPD cases caused by pneumococcal serotypes included in the vaccines, other serotypes, not included in the vaccine, are found to replace the serotypes in colonization and pneumococcal disease [54, 55]. In Sweden, 36% of the children between 1 and 5 years were colonized with pneumococci before the introduction of pneumococcal vaccines. After the pneumococcal vaccines were included into the childhood immunisation programme, the carriage rate was not severely different with 30%, which is attributed to serotype replacement [53].

In order to produce a vaccine that targets all pneumococcal serotypes simultaneously, several studies are concentrating on virulence factors present in all isolates. For example, the pneumococcal surface protein A (PspA), the pneumococcal surface antigen A (PsaA), as well as pneumolysin have been shown to mediate immunity against pneumococcal infections (See chapter 1.3.2) [56-58]. Moreover, immunization experiments with the pneumococcal surface protein C (PspC) were also shown to protect against colonization [59]. Increased protection rates have been observed, if these antigens were combined in immunization studies [57].

1.2 The immune system

The function of the immune system is to distinguish "self" from "non-self" and evading pathogens from non-pathogens, in order to prevent an infection, remove tumour cells and maintain an immune homeostasis. The immune system consists of lymphatic organs, physical barriers, cells and soluble mediators and can be divided into the innate and the adaptive immune response.

The innate immune system comprises epithelial barriers, e.g. skin and mucosa in the respiratory or gastrointestinal tract, cells, like macrophages, neutrophils or dendritic cells (DCs), and soluble components, like the complement system and antimicrobial peptides (AMPs). It is the first line of defence during an infection, with a rapid and non-specific response that is not adapted to the type of pathogen. Moreover, it regulates the adaptive immune response, e.g. by producing cytokines. In contrast to the innate immune response, the adaptive response is highly specific, adjusted to the pathogen and able to develop an immunological memory. Its function is mediated by B- and T-lymphocytes and soluble factors, like antibodies.

1.2.1 The innate immune response

Detection of invading pathogens and production of inflammatory mediators

The innate immune system is activated by pathogen-associated molecular patterns (PAMPs), structures, which are part of microbial pathogens and are required for their virulence. Usually, they are common to many different pathogens. Examples of PAMPs are bacterial DNA and lipopolysaccharide (LPS) [60]. PAMPs bind to and activate pattern-recognition receptors (PRR) on or inside host cells, e.g. Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) or cytosolic DNA receptors.

To date, we know of 10 human and 13 murine TLRs. While TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 are positioned on the plasma membrane, TLR3, TLR7, TLR8 and TLR9 are located in endosomes. TLRs consist of an ectodomain, required for the recognition of PAMPs, transmembrane domains, and Toll-interleukin 1 (IL-1) receptor (TIR) domains. There are different adapter molecules, known to interact with the TIR domain of TLRs, and which are required to evoke a response by the host cell. All TLRs, except for TLR3, known to recognize double-stranded ribonucleic acid (dsRNA), use the myeloid differentiation factor 88 (MyD88) as an adapter molecule to induce the transcription of inflammatory cytokines by activating the transcription factor nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs). TLR3, and also TLR4, use a TIR-domain-containing adapter

protein-inducing interferon-β (TRIF) as an adapter molecule to stimulate the transcription factors interferon regulatory factor 3 (IRF-3) and NF-κB, leading to the induction of type I interferon (IFN) and inflammatory cytokines, like interleukin-6 (IL-6) or tumor necrosis factor α (TNFα) [61, 62].

NLRs are located in the cytoplasm of cells. To date, we know of at least 22 different types in humans, and 33 in mice. NLRs consist of C-terminal leucine-rich repeats that recognize PAMPs, a nucleotide-binding oligomerization domain (NOD) and a variable N-terminal protein-protein interaction domain, e.g. caspase recruitment domain (CARD), that is crucial for inducing downstream signals, like NF-κB activation, leading to the transcription of pro-inflammatory cytokines [63]. Inflammasomes, consisting of nod-like receptor protein 3 (NLRP3) and absent in melanoma 2 (AIM2), are intracellular protein complexes that are activated upon bacterial infections. They recruit apoptosis-associated speck-like protein containing CARD (ASC), which binds to caspase-1 and evokes cleavage of the pro-forms of IL-1β and IL-18, thus leading to their maturation [62, 64].

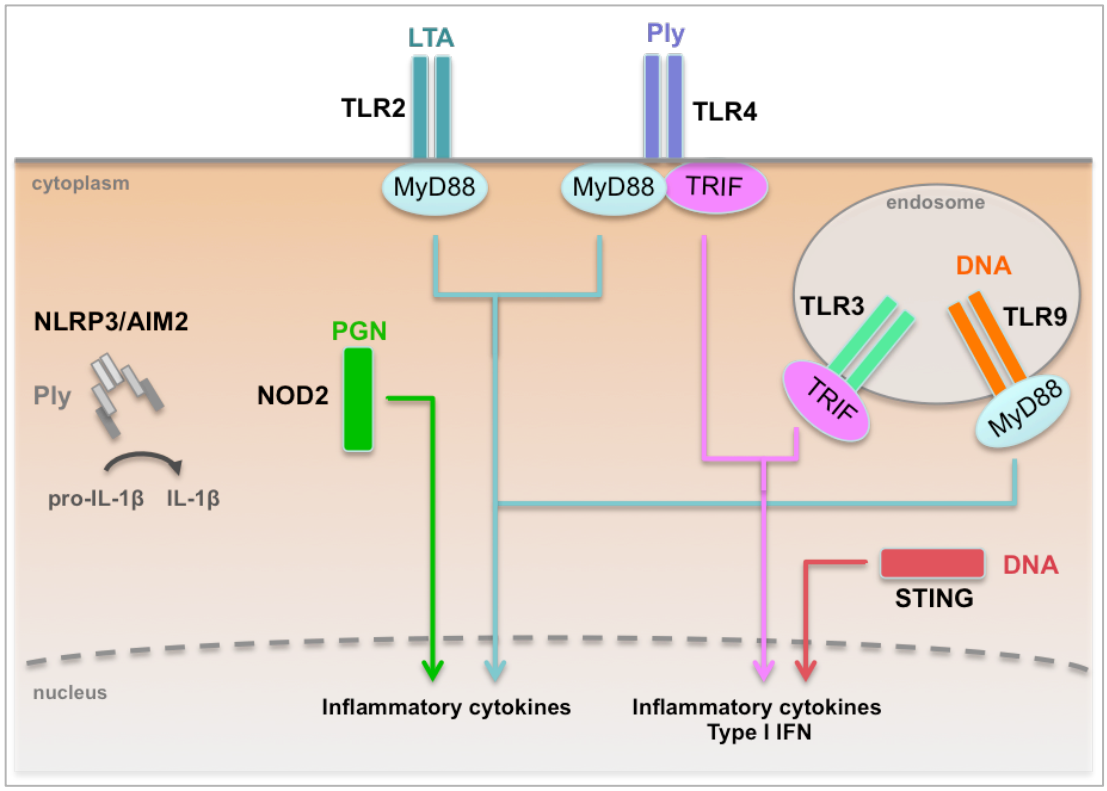

Figure 3 Schematic representations of selected PRRs and their known pneumococcal PAMPs. Pneumococcal LTA is sensed by TLR2, while TLR4 has been proposed to recognize Ply. The endosomal TLR9 senses DNA. TLRs that use MyD88 as an adapter (TLR2, TLR4, TLR9) induce the transcription of inflammatory cytokines, TLR3 and TLR4 signal via TRIF and thus activate additionally the expression of type I IFN. Ply is able to activate the NLRP3/AIM2 inflammasome and hence leads to the production of IL-1β. PGN activates NOD2 and the transcription of inflammatory cytokines, while DNA that signals via STING additionally induces type I IFN.

Various pneumococcal structures can be sensed by PRRs. Lipoteichoic acid (LTA), a component of the pneumococcal cell wall, is sensed by TLR2 [65]. Unmethylated CpG motifs in pneumococcal DNA can be recognized by TLR9 [66], and the pneumococcal toxin pneumolysin (Ply) has been suggested to be sensed by TLR4 [67]. Peptidoglycan (PGN), another pneumococcal cell wall component, can be sensed by NOD2, dependently on the activity of the pneumococcal toxin pneumolysin [68, 69]. Moreover, pneumolysin is capable of activating the inflammasome and subsequent production of IL-1β and IL-18 [70, 71] and is also required for the pneumococcal activation of the cytosolic DNA receptor stimulator of interferon genes (STING) [72] (Figure 3).

Macrophages

Bone marrow-derived monocytes, which circulate in the blood, are recruited and differentiate into macrophages and DCs during an inflammation [73]. Macrophages play a central role in the immune response and are present in many tissues and organs, especially those, which are exposed to the environment [74]. In the lungs, residential alveolar macrophages (AMs) represent 95% of all cells, and are therefore the major cell type [75].

The key function of macrophages is the phagocytosis and killing of particles and pathogens, both opsonized and non-opsonized [75, 76]. Invading microbes can be recognized and taken up into the cell due to binding to different receptors on the cell surface, like C-type lectins (e.g. mannose receptor), scavenger receptors (e.g. macrophage receptor with collagenous structure (MARCO)), complement receptors (CR) or immunoglobulin receptors (Fcγ receptors) [77, 78].

Following phagocytosis, pathogens are eradicated by reactive oxygen species (ROS) or reactive nitrogen species (RNS), e.g. superoxide, hydrogen peroxide (H_2O_2) or nitric oxide [79]. Furthermore, host cells secrete pro-inflammatory cytokines and chemokines, like IL-8, which recruit neutrophils to the site of infection, and the production of monocyte chemoattractant protein-1 (MCP-1) or RANTES evokes an influx of activated monocytes and lymphocytes [75].

Macrophages play a significant role in fighting pneumococcal pneumonia and the importance of several receptors in pneumococcal uptake has been described in the literature. Scavenger receptors, such as MARCO [80], C-type lectins, such as SIGN-R1 [81], or the complement receptor CR3 [82] have been shown to mediate pneumococcal phagocytosis. Pneumococci, on the other hand, evade phagocytosis mechanisms by the host with the help of their thick polysaccharide capsule [83]. Although the production of ROS has been implicated in macrophage-mediated killing of different bacteria, pneumococcal clearance by macrophages is facilitated by RNS, and not attributed to ROS production [84].

Neutrophils

Neutrophils are produced in the bone marrow and are afterwards released into the vascular system, where they represent 60% of all leukocytes in humans [85]. During inflammation in the lungs, macrophages and epithelial cells produce cytokines and chemokines, which recruit neutrophils into the alveolar space [86, 87]. Like macrophages, neutrophils phagocytose bacteria and eradicate them with the help of ROS, but also non-oxidative mechanisms, like AMPs, proteases and the formation of neutrophil extracellular traps (NETs) [88, 89].

As mentioned above, *S. pneumoniae* was shown to avoid phagocytosis and to be resistant to ROS. Instead, neutrophils fight pneumococcal infections with serine proteases [90, 91]. In order to escape killing by neutrophils, pneumococci have developed different immune evasion strategies. The pneumococcal capsule was shown to promote escape from NETs [92], and pneumococcal endonuclease A degrades DNA, the main building block of NETs [93].

Dendritic cells

DCs account for only 1% of all immune cells [94]. They originate in the bone marrow, and represent the connection between the innate and the adaptive immune response. These cells express a number of receptors, like scavenger, complement receptors or Fc receptors, which promote phagocytosis and are thus required for antigen presentation, the most important function of DCs [95].

DCs are located in the mucosa, where they constantly collect antigens. After maturation and translocation to the lymph nodes, they present these antigens to T-cells via major histocompatibility complex class II (MHC class II) molecules, leading to the activation of T-cells and the initiation of an adaptive immune response [96].

Antimicrobial peptides and collectins

AMPs are effective against Gram-positive and Gram-negative bacterial, viral, as well as fungal pathogens and show a great variation in size (6 - 59 amino acids), sequence and secondary structure. They bind to microbes and kill them either by disturbing membrane integrity and causing lysis, or they enter the cell and interfere with essential mechanisms, e.g. nucleic acid synthesis [97].

In the respiratory tract of humans, primarily epithelial cells and neutrophils produce AMPs, which help to kill invading pathogens [98]. Neutrophil α-defensins / human neutrophil peptides (HNPs), human β-defensins (hBDs), and cathelicidins, like hCAP18/LL-37 are the major AMPs present in the lungs and bronchoalveolar lavage (BAL) (see Table 2) [99].

Table 2 AMP-producing cells in the respiratory tract of humans [99, 100]

Cell type	AMPs	Function
Neutrophils	α-defensins (HNP 1-4)	Microbial killing
Epithelial cells	hBDs	Microbial killing Chemo-attraction of immune cells Activation of DCs
Neutrophils Epithelial cells	hCAP18/LL-37	Microbial killing

Some hBDs, like hBD-1, are constitutively expressed, while other hBDs are induced e.g. by TLR-signalling or cytokine expression [100]. LL-37 is shown to be resistant against proteolytic activity due to its ability to form aggregates [101]. Its expression is regulated by inflammatory signalling, Vitamin D and endoplasmic reticulum stress. The murine homologue of LL-37 cathelicidin-related antimicrobial peptide (CRAMP) [102] and several defensins have been identified in mice [103], which are suggested to play an important role in murine host defence mechanisms in the lung [104].

Pneumococci are sensitive to treatment with LL-37 from mast cells [105] and human α-defensins produced by neutrophils [106]. Furthermore, primary human lung epithelial cells have been shown to secrete hBD-2 and hBD-3 in response to pneumococcal infections, which promoted bacterial clearance [107].

In the lungs, surfactants are secreted by type II alveolar epithelial and Clara cells [108] and consist of 90% lipids and 10% proteins. They play an important role in reducing surface tension, and take part in the innate immune response. There are several surfactant proteins (SP). This thesis includes SP-A and SP-D, which are hydrophilic and belong to the group of collagen-containing C-type lectins (carbohydrate-binding proteins), so-called collectins. SP-A is believed to stabilize surfactants, while SP-D is involved in keeping the homeostasis. Moreover, both proteins opsonize pathogens, promoting their clearance [109, 110].

Recombinant SP-D was found to bind to and agglutinate pneumococci, but did not promote neutrophil-mediated killing [111]. Using an *in vivo* model of intranasal challenge, it was demonstrated that SP-D endorses pneumococcal clearance and prevents bacterial translocation from the upper to the lower respiratory tract [112]. SP-A, on the other hand, was shown to promote pneumococcal uptake by alveolar macrophages via the scavenger receptor [113].

1.2.2 The adaptive immune response

T-lymphocytes

Naïve T-lymphocytes (or T-cells) originate in the thymus and are found in the blood and lymphatic organs. T-cell receptors on their surface recognize antigens which are presented via MHC molecules, e.g. by DCs. This leads to the activation of the T-cells, evoking either a cytotoxic function or the production of cytokines. Cytotoxic T-cells (or CD8+ T cells) are activated by MHC class I molecules and have the ability to directly kill infected cells, e.g. by lysis. CD4+ helper T-cells, on the other hand, are activated by MHC class II molecules and produce cytokines, which can be lethal to infected cells, and lead to immunoglobulin (Ig) production by B-cells or stimulate other T-cell functions [114].

After an infection with *S. pneumoniae*, DCs produce large amounts of IL-12, a pro-inflammatory cytokine that promotes the differentiation of helper T-cells (T_H-cells). Several studies have elucidated the importance of IFN-γ secretion by T_H-1 cells in *in vivo* models after pneumococcal challenge [115-117].

B-lymphocytes

B-lymphocytes (or B-cells) mature in the bone marrow and play a major role in the adaptive immune response. B-cells bind and take up antigens, in order to present them on their surface by MHC class II molecules. These antigens can now be recognized by specific T_H-cells, which in turn stimulate the differentiation of B-cells into plasma cells producing high-affinity class-switched antibodies (IgG, IgE or IgA) or memory B-cells [118, 119].

Carbohydrates alone, like the pneumococcal vaccine PPSV23, activate B-cells in a T-cell independent manner, since they cannot be presented by MHC class II. Therefore, B-cells are only capable of producing short-lived low-affinity antibodies, like IgM. Subsequently, the immune response does not last long. Furthermore, children under the age of 2 years are not able to evoke an immune response to carbohydrates only, since their B-cells are not fully matured. They hardly express type 2 complement receptors and lack certain cytokines, needed to activate B-cells. In order to induce an immune response in very young children, the pneumococcal vaccines PCV7, PCV10 and PCV13 were designed differently. The pneumococcal polysaccharides have been linked to a carrier protein, which can be presented by MHC class II molecules and this leads to a T-cell dependent immune response [118].

1.3 Pneumococcal-Host interactions

Carbohydrates are essential structures to enable pneumococcal interactions with the host. They facilitate pneumococcal colonization and invasive disease but also evoke a host immune response. The following chapter will discuss host glycosylation, with an emphasis on sialic acids (Sias), the carbohydrates that are found on the tip of most glycan strands. In order to hide and escape from the host's immune system, pneumococci comprise several virulence factors, of which the pneumococcal capsule, the sialidase NanA, the main Sia transporter SatABC, the two-component system CiaRH, the streptococcal pyruvate oxidase SpxB, the serine protease HtrA, and pneumolysin will be described. Moreover, the pathogenesis of influenza/pneumococcal coinfections will be discussed.

1.3.1 Host glycans and their biological functions

Host glycosylation

In 1967, Rambourg and colleagues observed that the cell surface is highly covered with sugar molecules, the glycocalyx [120]. Glycans exist in many variations and are built of mono- or oligosaccharides, which are covalently linked to a non-carbohydrate moiety, a protein or lipid, or exist freely [121]. The most common monosaccharides present in mammalian glycoconjugates are listed in Table 3.

Table 3 The most common monosaccharides in mammalian glycans [121]

Name	Structure	Examples
Sialic acids	Nine-carbon backbone acidic sugar	*N*-acetylneuraminic acid, *N*-glycolylneuraminic acid
Hexoses	Six-carbon neutral sugars	Glucose, galactose, mannose
Hexosamines	Amino group at position 2 of an hexose which is free or *N*-acetylated	*N*-acetylglucosamine, *N*-acetyl-galactosamine
Deoxyhexoses	Six-carbon neutral sugar without hydroxylgroup at position 6	Fucose
Pentoses	Five-carbon sugar	Xylose
Uronic acids	Hexose with negatively charged carboxylate at position 6	Glucuronic acid, Iduronic acid

Glycosylation is the most common post-translational modification of proteins. Depending on their linkage to a protein, the aglycone, glycans are mainly divided in to N- and O-linked. N-linked glycans are covalently attached to a polypeptide via an asparagine (Asn) residue. O-linked glycans are linked to the polypeptide via *N*-acetylgalactosamine (GalNAc) on a serine (Ser) or threonine (Thr) residue (Figure 4). In eukaryotes, these glycosylations take place in the cytoplasm, the endoplasmatic reticulum and golgi [121, 122].

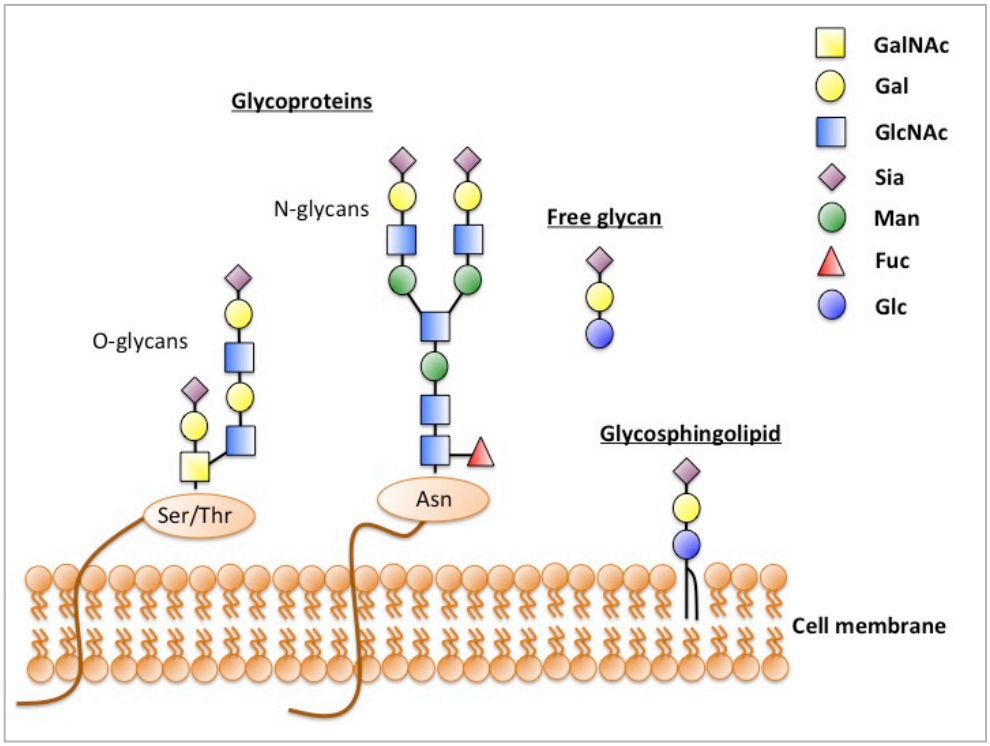

Figure 4 Schematic representations of common mammalian glycans. Glycoproteins and glycolipids are usually decorated with chains of GalNAc, galactose (Gal), *N*-acetylglucosamine (GlcNAc), mannose (Man), fucose (Fuc) or glucose (Glc) molecules, with a terminal Sia molecule. Modified after Varki (2007) [123].

Other life forms such as plants and bacteria differ greatly from mammals in their oligosaccharide composition. To date, several glycans are exclusively found in bacteria, e.g. 3-deoxy-D-manno-oct-2-ulosonic acid (KDO), presenting part of the core of the bacterial endotoxin LPS, and *N*-acetylmuramic acid (MurNAc), found in peptidoglycan, the main building block of the bacterial cell wall [124].

Sialic acids

The biochemists Blix and Klenk discovered Sias in glycolipids of the brain and in salivary mucins [125, 126]. The terminal monosaccharide of glycoconjugates, like glycoproteins and glycolipids, is usually a nine-carbon backbone sugar, called neuraminic acid or Sia, with

modifications mainly occurring at position 4, 5, 7, 8 and 9 [121, 127]. Sias are found on all cells in animals of the deuterostome lineage (from sea urchins to mammals) [123, 128], and in some bacteria, fungi and protozoa [129]. Although Sias are usually not present in insects, they have been detected in early developmental stages of *Drosophila melanogaster* and the cicada *Philaenus spumarius* [130, 131].

To date, there are about 50 different types of Sia known in nature. Neu5Ac (*N*-acetylneuraminic acid) and Neu5Gc (*N*-glycolylneuraminic acid) are the most common ones [128]. Most of them are attached by their 2-carbon to the 3- or 6-carbon of Gal or GalNAc underneath, commonly referred to as α2-3- or α2-6-linkage [121].

CMP-NeuAc hydroxylase (CMAH)

In most animals, the enzyme CMP-Neu5Ac hydroxylase (CMAH) transforms CMP-Neu5Ac into CMP-Neu5Gc by adding a single oxygen atom [132]. About 3 million years ago, a 92-bp deletion in the exon 6 encoding *CMAH* occurred in humans. Consequently, humans are only able to synthesize Neu5Ac, while many other mammals, including our closest relatives the chimpanzees, present mainly Neu5Gc as terminal sugar on their glycoconjugates (Figure 5) [133, 134]. The occurrence of the *CMAH* mutation was predicted to have happened when stone tools were used to butcher hunted animals, which might have caused minor injuries and infections [135]. It was suggested that evolutionary pressure caused by Neu5Gc-recognizing pathogens and higher fertility of Neu5Gc-negative females led to the positive selection of CMAH-negative individuals [136, 137].

Noteworthy, humans are not the only species with a non-functional CMAH. Recently, it was described that New World Monkeys obtained a mutation in their *CMAH* gene already 30 million years ago [138]. Likewise, ferrets have a mutation in *CMAH* in their genome, which was shown to increase their susceptibility to human IAV [139].

Different human-adapted pathogens have evolved their virulence mechanisms in accordance to the human Sia profile. The merozoit of *Plasmodium falciparum* recognizes Neu5Ac, but not Neu5Gc [140], therefore infecting New World monkeys but no other primates [141]. Similarly, the typhoid toxin of *Salmonella* Typhi was shown to strongly bind to Neu5Ac, but only weakly to Neu5Gc [142].

The loss of Neu5Gc is shown to affect B-cell reactivity in *Cmah*$^{-/-}$ mice, since its absence leads to enhanced levels of B-cell proliferation and antibody production [143]. Similarly, T-cells of *Cmah*$^{-/-}$ mice exhibit a greater activation upon stimulation compared to wt cells, which can be repressed by Neu5Gc-treatment [144]. Moreover, in comparison to chimpanzees, humans display a more active immune system. Both B- and T-lymphocytes of humans are reported to react stronger to stimuli than those of chimpanzees [145].

Although humans are not able to synthetize Neu5Gc, this Sia can be incorporated into human tissues through their diet. Neu5Gc is antigenic in humans, evoking an antibody response [146], which was suggested by Hedlund *et al*. to promote inflammation and the development of cancer [147] and later experimentally confirmed by Samraj *et al*. using *Cmah*$^{-/-}$ mice [148].

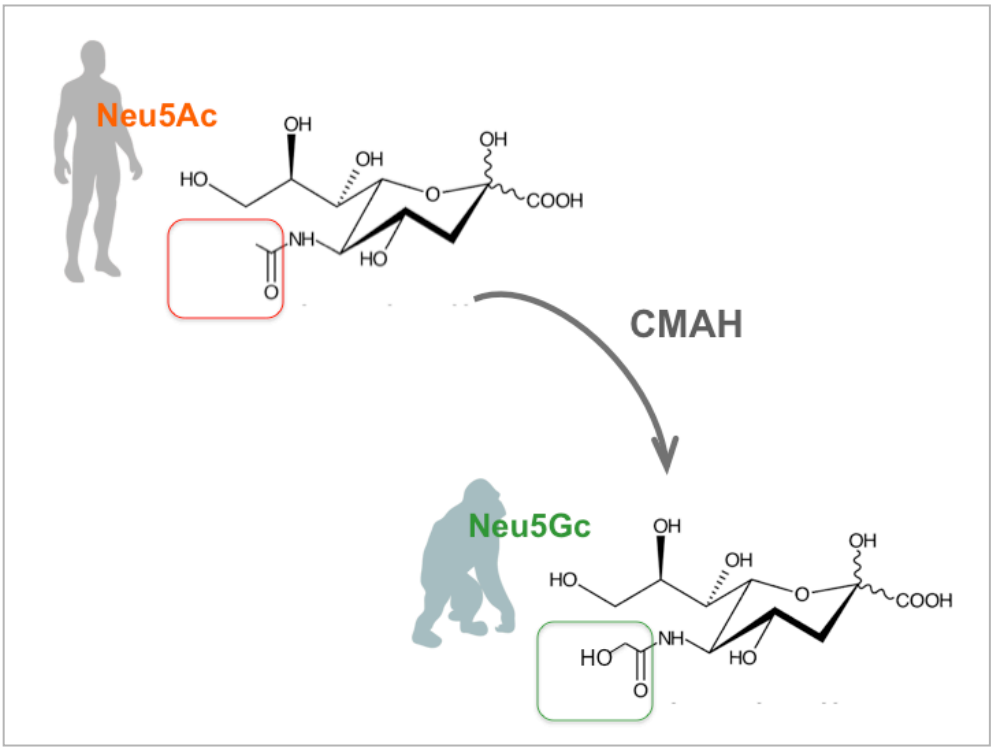

Figure 5 Humans have a deletion in the gene encoding CMAH. Due to the inactive CMAH, human cells only produce Neu5Ac. Most other mammals, including non-human hominids like gorillas or chimpanzees, convert Neu5Ac into Neu5Gc. Both molecules differ in a single oxygen atom (framed).

Role of glycosylation in the host

The majority of glycans are indispensible in biological systems and their roles are very divers. Therefore, this chapter only highlights some of the most important features.

Glycans define the physical characteristics of many structures, like plant cell walls or exoskeletons of insects [149, 150]. Moreover, glycosylation is required for correct folding of glycoproteins [151], and to protect them from cleavage by proteases [152]. Sias are fundamentally important for embryonic development, as the disruption of their biosynthesis is lethal in a mouse model [153]. Negatively charged Sias on the cell surfaces lead to the electrostatic repulsion and thus prevent non-specific interactions of cells, e.g. of red blood cells in circulation [154]. In addition, they are important for cell-cell interactions, like the binding of P-selectins (Calcium-dependent lectins) on endothelial cells and glycoproteins on

the surface of neutrophils, which enables neutrophil adhesion to endothelial cells and their “rolling" as part of inflammatory processes [155].

The most important function of glycans, e.g. Sias, is probably to distinguish ‘self’ from ‘non-self’. By covering receptors and antigenic proteins, glycans are recognized as self-associated molecular patterns (SAMPs), which prevent immune responses against host-derived structures [156]. Several members of the family of CD33-related Sia recognizing immunoglobulin superfamily lectins (CD33r Siglecs) have been shown to be involved in the prevention of immune responses. CD33rSiglecs are transmembrane receptors that consist of extracellular sialic-acid binding domains, immunoglobulin domains and often of at least one cytosolic immune-receptor tyrosine-based inhibitory motif (ITIM), which inhibit cell activation and cytokine signalling when the ligand is bound [157].

In contrast, asialoglycoproteins, desialylated structures, are recognized by the hepatic Ashwell-Morell receptor, which removes them from circulation [158]. This is of importance in the clearance of e.g. senescent platelets. These blood components take part in coagulation and regulate their own elimination with the help of neuraminidases, which desialylate their surface-located glycan structures in order to evoke their clearance [159].

Role of glycans during infection

Many pathogens enter the host via inhalation into the respiratory tract and encounter epithelial surfaces, which are usually covered by a thick and viscous layer, consisting of high molecular weight glycoproteins with many clustered O-linked glycans, the so-called mucins, water, salts, lipids and other highly glycosylated proteins [160, 161]. The mucus traps microbes and particles, which are cleared by cilia movement or coughing [162]. Thus the mucus presents a physical barrier to inhibit invading pathogens.

However, infections and inflammations have been shown to alter glycosylation of the host. One example is the sialylated LewisX blood group antigen. It plays an important role during inflammation [163], as it is upregulated in gastric tissues during *Helicobacter pylori* infections [164], as well as in the respiratory mucins of cystic fibrosis patients [165].

Microbes interact with glycans on the surface of host cells, which promotes pathogenesis [166]. During pneumococcal colonization, adhesion to glycosaminoconjugates, like heparan sulfate, presents one of the first steps in the interaction with epithelial cells [167]. Furthermore, pneumococci engage with the host glycocalyx and produce enzymes and transporters to scavenge and take up Sias (see chapter 1.3.2), but also other sugars, like Gal and GlcNAc, of the glycan strand [168, 169]. In pneumococci, Sia has been shown to promote biofilm formation, nasopharyngeal colonization and bacterial outgrowth from the upper to the lower respiratory tract [170-172]. While intranasal administration of Neu5Ac

enhanced pneumococcal colonization and spread to the lungs and brain of mice, Neu5Gc did not have any effect on pneumococcal carriage or disease [170, 173].

Viruses, like IAV, also interact with Sias on host cells in order to facilitate their entry [35]. Human IAV adheres preferentially to α2-6-linked Sias [174], which are present on ciliated cells in the respiratory tract [175].

Interestingly, bacteria have developed strategies to evade the host's immune response by mimicking host glycans or by decorating themselves with Sias. While some bacteria are able to synthesize Sia on their own (e.g. *Escherichia coli* K1 or *N. meningitidis*), others, like *N. gonorrhoeae*, scavenge Sias from host substances and sialylate their surfaces with the help of sialyltransferases [129].

The capsule of group B streptococci (GBS) is shown to contain Sias and represents a major virulence factor [176]. By binding to the CD33r Siglec-9 on neutrophils, the capsular Sia of GBS downregulates its own killing [177]. In contrast, the pneumococcal capsule does not contain Sias, but pneumococci express sialidases, which cleave terminal Sias from the glycan strand [178]. The pneumococcal sialidase NanA has been associated with an unmasking of the CD33r Siglec-5 and subsequent induction of an immune response in macrophages [179]. During sepsis, NanA removes terminal Sias from glycoconjugates of platelets, promoting intravascular coagulation. The Ashwell receptor clears desialylated platelets from the circulation and therefore improves the outcome of this complication [180].

As discussed earlier, PRRs, e.g. TLRs or NLRs recognize PAMPs. Such ligands are often glycan-containing structures, e.g. bacterial LPS or peptidoglycan, or nucleic acids, like bacterial and viral DNA or RNA [181], which play a significant role in host-microbe interactions.

Sialic acid catabolism in bacteria

Since glucose is not detectable in the respiratory tract of healthy individuals [182], pulmonary pathogens may utilize Sia in order to gain energy in form of nitrogen or carbon [183]. Studies in *E. coli* have shown that Neu5Ac is transported into the bacterial cell and catabolized, leading to the generation of pyruvate and *N*-Acetylmannoseamine (ManNAc), which is, via several steps, converted to Fructose-6-Phosphate, which is part of the glycolysis [184, 185]. Moreover, it was shown that *E. coli* is also able to grow on Neu5Gc as sole carbon source by using the same enzymes as for the catabolism of Neu5Ac [186].

S. pneumoniae was predicted to possess all genes required for Sia metabolism [187, 188]. However, in contrast to TIGR4, D39 is not able to ferment Sia due to a frameshift in the gene for the neuraminate lyase [168].

Other serotypes, like 19F and 23F, are shown to be able to grow on Sia Neu5Ac as sole carbon source. As compared to the presence of glucose, growth on ManNAc or Neu5Ac only leads to a much prolonged generation time [168, 172, 189].

1.3.2 Pneumococcal virulence factors

Capsule

Pneumococci are surrounded by a thick polysaccharide capsule, which presents a major virulence factor and is the target for current vaccines on the market [4]. Non-encapsulated isolates seldom cause IPD in humans [190] and are significantly attenuated in their virulence using mouse models [191]. The composition of the capsule varies greatly between serotypes [4], and affects the invasive disease potential of the bacteria, although other bacterial factors are also involved [192]. Since most capsular types are negatively charged, electrostatic repulsion prevents pneumococci from being trapped in the mucus and from phagocytosis by immune cells [83]. Moreover, the capsule shields surface proteins and protects pneumococci from recognition and opsonisation by immune cells [193].

Pneumococci of the same serotype can show substantial variations in their capsular thickness, and are therefore divided into opaque and transparent variants. The switch in capsule production, also known as phase variation, allows pneumococci to adapt to different body sites, like the mucosa of the respiratory tract or the blood stream. In contact with epithelial cells, pneumococci produce lower amounts of capsular polysaccharide in order to expose adhesive structures on their surface [194]. These transparent variants were shown to establish a robust colonization in an infant-rat model [195], but hardly caused any sepsis in a mouse model after intraperitoneal challenge. In contrast, all mice succumbed to an intraperitoneal infection with opaque isolates, which produce large amounts of capsule and reduce the detection by the immune system [196].

Virulence factors involved in sialic acid scavenging and uptake

S. pneumoniae is able to use Sia as carbon source [172, 197]. Genes for Sia removal, uptake and metabolism are encoded in two loci, the *nanAB* and *nanC* loci [178, 184]. These genetic regions harbour up to three different sialidases, with NanA, NanB and NanC to be common to 100%, 96% and 51% of all isolates, respectively [198].

The *nanAB* locus is predicted to contain four transcriptional units [178] and catabolite repression elements, which lead to its transcriptional inhibition in the presence of glucose

[199]. In the absence of glucose, genes within this locus are upregulated in response to Neu5Ac [189, 200].

This thesis focuses on NanA, which is a typical sialidase and scavenges Sias from host glycoconjugates [169, 201]. NanA is located on the bacterial surface and linked to the peptidoglycan cell wall via an LPxTG motif in most pneumococci [202]. Interestingly, in *S. pneumoniae* TIGR4 NanA is secreted due to an authentic frameshift [188].
Despite comprising an enzymatic active part, NanA also consists of a carbohydrate-binding domain. Using a model of experimental meningitis, it was demonstrated that the lectin-like domain is of higher importance than the sialidase domain to achieve adhesion to and invasion into brain endothelial cells, as well as to activate an immune response in these cells [203, 204].

NanA is able to remove both α2-3- and α2-6-linked Sias from the underlying Gal of the glycan strand [178]. By removing terminal Sias from epithelial cells, potential binding sites are unmasked, and pneumococcal adhesion is promoted [205]. Additionally, desialylation of THP-1 monocytes is reported to stimulate an immune response and to induce cytokine secretion [179]. Moreover, it was suggested that desialylation of host factors, e.g. lactoferrin, interferes with their functionality [178], thus promoting pneumococcal immune evasion. *S. pneumoniae* has also been demonstrated to desialylate host structures of other bacteria in the respiratory tract, like *N. meningitidis* and *H. influenzae,* which is proposed to provide a competitive advantage for pneumococci [206].

NanA expression is increased in transparent compared to opaque variants [178], and it was shown to be beneficial during colonization in a chinchilla model [201]. Moreover, *nanA* transcription was strongly increased in bacteria isolated from the nasopharynx, lungs and brain of mice in comparison to blood [207, 208].
Several groups observed that NanA is also required for colonization of the nasopharynx and lungs using a murine model of intranasal challenge [209, 210]. Moreover, a recent study reported that NanA-mediated exposure of Gal *in vivo* in the nasopharynx promotes pneumococcal biofilm formation [211]. During sepsis, NanA is demonstrated to be redundant for pneumococcal survival in the vascular system [210, 212, 213], but there are studies showing a reduced virulence of NanA-deficient mutants in the blood stream [209, 213]. Moreover, NanA has been shown to promote pneumococcal meningitis in murine *in vivo* models [203].

Three different Sia transporters have been predicted in the *nanAB* and *nanC* loci of *S. pneumoniae* TIGR4, a solute symporter SP1328, and two ABC transporters SP1688-90 and SP1681-83 [187]. The latter was identified as the main Sia transporter, and named SatABC, Moreover, it has been shown to play an important role in nasopharyngeal colonization of mice [172].

Two-component system CiaRH

Two-component systems (TCS) allow bacteria to sense and respond to environmental signals. They comprise a membrane-bound histidine kinase (HK), and a cytoplasmic response regulator (RR). The HK detects the signal on the outside of the cell, is autophosphorylated and phosphorylates the RR, which alters its conformation in order to bind to specific promoter regions and thus regulates transcription [214].

In *S. pneumoniae*, 13 different TCS and a single RR have been annotated, of which the majority affects pneumococcal virulence [215]. During the analysis of mutants resistant to the β-lactam antibiotic cefotaxime, TCS05, also known as CiaRH, was identified and a connection with the pneumococcal cell wall machinery was suggested [216]. Besides sensitivity to cefotaxime, CiaRH was also shown to affect autolysis. Deletion mutants in *ciaR* lyse quickly upon entry into stationary phase or in response to cell wall inhibitors, like vancomycin [217]. Other studies revealed that CiaR regulates the expression of several genes involved in biosynthesis of the pneumococcal cell wall [218, 219].

The use of microarrays and solid-phase DNA binding assays also identified the high-temperature requirement A gene (*htrA*) to be regulated by CiaRH [218, 219] (see below), and the contribution of CiaRH to oxidative stress resistance and virulence in rodents, using nasopharyngeal colonization and pneumonia models, was demonstrated [215, 219, 220]. Moreover, CiaRH was shown to play a role in bacteriocin production [218].

A mutation in the HK CiaH that mimicked its activation, led to the complete abolishment of competence [216], while a mutation in RR CiaR restored competence [221]. Another study reported the upregulation of the competence operon in a *ciaRH*-mutant [219]. However, a direct interaction between CiaR and genes involved in competence could not be observed [218]. Moreover, CiaR expression is also regulated by competence, as *ciaR* transcription is increased in the late phase of competence, which was suggested to promote re-entry into the non-competent state [222].

The external stimulus for the activation of CiaH is not known yet, but it might be induced upon stress. Alignments of promoter sequences targeted by CiaR identified its binding motif (NTTAAG-N5-TTTAAG) [223]. Moreover, by cloning CiaR-regulated promoter regions in front of a β-galactosidase gene (*lacZ*), it was shown that the position of the binding motif affects transcription control. If the motif is located upstream of the transcriptional start site, CiaR will positively regulate gene transcription. In contrast, if the binding site is located on the other DNA strand inside the transcribed region, CiaR downregulates promoter activity [224].

Bioinformatic analysis identified five cia-regulated small RNAs (csRNA). Their functional analysis implicated a role in autolysis, although the phenotype that was caused by their

deletion was not as pronounced as in a *ciaR* null mutant [224]. Moreover, a function in competence [225, 226] and virulence in mice after intranasal challenge was suggested [227].

CiaRH is conserved in streptococci but absent in other bacterial species [223]. In order to bind to promoter fragments and to regulate transcription, CiaR needs to be phosphorylated. Interestingly, depending on the growth media used, CiaH is or is not required to activate CiaR [221, 228]. Intracellular acetyl phosphate (AcPh) production is strongly regulated by the streptococcal pyruvate oxidase (SpxB) (see below) [229]. Deletion of SpxB leads to a significant downregulation of CiaR-regulated genes, suggesting that intracellular AcPh is capable of phosphorylating this RR [230].

Streptococcal pyruvate oxidase (SpxB)

The pneumococcal pyruvate oxidase SpxB is located in the cytoplasm and catabolizes the decarboxylation of pyruvate leading to the production of AcPh and H_2O_2 [231]. Pneumococci generate high concentrations of H_2O_2, which enables them to outcompete other respiratory pathogens, like *N. meningitidis* or *H. influenzae,* present in the same niche [232]. Interestingly, pneumococci themselves lack catalase [233]. Instead HtrA, as mentioned above, was shown to promote resistance to oxidative stress [234].

SpxB has been associated with the pneumococcal capability to bind to glycoconjugates, which are found on endothelial cells and lung epithelial cells [231]. A mutation in *spxB* leads to drastically reduced AcPh and acetyl-CoA amounts in the bacteria, which are suggested to mediate adhesin expression. On one hand, acetyl-CoA is required for the biosynthesis of fatty acids, amino acids and the cell wall, and subsequently affects fundamental structures of the bacterial cell [231]. On the other hand, AcPh is known to act as a phospho-donor for bacterial TCS [235]. A deletion of *spxB* downregulates *ciaR* mRNA levels and thus CiaR- regulated genes [230], which might affect adhesin expression.

H_2O_2 has been shown to inhibit ciliary beating and cell integrity of human nasal epithelial cells [236]. It was also reported to induce host cell apoptosis in meningitis models, using microglia cells and rat primary neurons [237], and in lung epithelial cells by introducing DNA double-strand breaks [238]. It has been reported to evoke stress responses in respiratory epithelial cells and the secretion of inflammatory mediators [239]. Moreover, a deletion of *spxB* in serotype 4 pneumococci reduces the production of capsular polysaccharides [240]. In summary, SpxB is a main pneumococcal virulence factor and its importance has been demonstrated using several *in vivo* models, e.g. of colonization, pneumonia or bacteraemia [231, 240].

High temperature requirement A (HtrA)

HtrA is a serine protease that is highly conserved among bacteria and located on the pneumococcal surface [241]. It plays an important role in protein quality control, as it is described to have chaperone and protease functions. HtrA promotes protein folding at lower temperatures, and fosters degradation of misfolded proteins at higher temperatures [242].

In pneumococci, HtrA promotes growth at higher temperatures [234] and its expression is regulated by the pneumococcal TCS CiaRH [218, 219]. Moreover, HtrA mediates pneumococcal resistance to oxidative stress [234], which is caused by ROS-producing immune cells like macrophages or neutrophils [243, 244].
Several reports have highlighted an effect on competence by HtrA, although they presented opposing results. Some studies have shown that HtrA contributes to the inhibitory effect of CiaRH on competence by degrading competence-stimulating peptides [241, 245], while others have demonstrated a decrease in competence in a mutant lacking HtrA [234].

HtrA of *H. pylori* and *Campylobacter jejuni* have been shown to degrade E-cadherin, which leads to the disruption of tight junctions, promoting bacterial invasion [246, 247]. In *S. pneumoniae*, HtrA has also been associated with virulence. Mutants in *htrA* showed lower colonization rates and led to lower bacterial counts in the lungs of rodents after an intranasal challenge, as well as a reduced rate of bacteraemia after intravenous infection [219, 234, 248].

Pneumolysin

Pneumococci were the first bacteria, which were demonstrated to lyse red blood cells [249]. This lysis is caused by pneumolysin, a major virulence factor, which is expressed by nearly all pneumococcal isolates [250]. It is a 52 kDa binding monomer that forms a ring of 30-50 subunits, thus interfering with membrane integrity and leading to cell lysis [251, 252].

Pneumolysin binds to cell membranes via cholesterol [251], but has also been reported to adhere to glycan structures. It was shown to attach to mannose and sialylated LewisX blood group antigens on the cell surface, since the haemolytic activity of pneumolysin was strongly inhibited after pre-incubation with these sugars [253, 254]. Sialyl LewisX is found on various immune cells like neutrophils or monocytes [255, 256] and has been shown to play a significant role in the first step of extravasation of these cells [163].

To interact with host cells, pneumolysin has to be present on the outside of bacteria. Interestingly, pneumolysin was found to be located in the cytoplasm [257] and does not contain a signal sequence, which would direct its translocation to the extracellular space [258]. Yet, it is detected non-covalently attached to the bacterial cell wall [259]. For many years, pneumolysin was thought to be released during pneumococcal autolysis. However,

recent studies demonstrated an autolysis-independent secretion of the cytotoxin, suggesting an active transport of pneumolysin out of the bacterial cell [260-262].

Pneumolysin activates the immune system. It promotes bacterial adhesion to epithelial cells [263] and reduces ciliary movement in the lungs [236]. At sublytical concentrations, pneumolysin stimulates the secretion of cytokines, like IL-6, TNFα or IL-1β, by different cell types, e.g. DCs and monocytes [71, 264]. However, other reports showed an inhibition of inflammatory responses by pneumolysin [265, 266]. While some studies demonstrated that host cells sense pneumolysin in a TLR4-mediated way, others observed TLR4-independent but inflammasome dependent signalling in response to this cytotoxin [71, 267]. Its importance *in vivo* was demonstrated using mouse models of pneumonia and bacteraemia, as a mutant deficient in pneumolysin was severely attenuated in virulence [250, 268]. Moreover, pneumolysin-mediated inflammation was shown to endorse pneumococcal shedding and its transmission between hosts [269].

Pathogenesis of coinfections with influenza and pneumococci

Preceding influenza infections significantly worsen the outcome of a secondary pneumococcal infection. The pathogenesis of coinfections with influenza and pneumococci is regulated by an interplay of viral, bacterial and host factors.

Human influenza viruses bind to α2-6-linked Sias [174], which are found in the respiratory tract of men [175]. The infection with the virus induces drastic changes in the respiratory tract, which promote pneumococcal infections. Influenza infections foster secondary bacterial colonization by damaging the respiratory epithelium, which prevents ciliary clearance [270]. Moreover, viral sialidases have been shown to cleave Sias from epithelial surfaces and mucins, thereby exposing host receptors, enabling increased bacterial adhesion [271, 272], and providing an energy source for pneumococcal growth and translocation from the upper respiratory tract into the lungs [171].

At early stages of influenza infections, the number of alveolar macrophages in the lungs are severely decreased [273]. It takes up to 2 weeks until the macrophage reservoir is restored by the infiltration of new macrophages. Thus, the host susceptibility to secondary bacterial infections is increased during this period [35]. Furthermore, influenza infections increase the production of IFN-γ, which is shown to inhibit macrophage function by downregulating MARCO, a scavenger receptor known to promote pneumococcal phagocytosis [274]. Following influenza infections, an inhibition of ROS-mediated bacterial killing by macrophages was also demonstrated [275].

Although macrophage numbers are decreased, neutrophil numbers are increased at day 7 post influenza infection. However, their role in bacterial influenza coinfections is still debated. While some studies report that increased inflammatory processes due to neutrophil influx

correlate with higher bacterial burden and lower survival rates in coinfected versus single infected mice, others described a redundancy of neutrophils in coinfection settings, as their depletion did not affect the disease outcome [276].

2 AIMS

Since glycans are highly abundant in nature, they play an important role in host-pathogen interactions. Especially Sias, the terminal sugars of the glycan strand, serve as receptor for many pathogens. The main scope of this thesis was to determine virulence regulation in the human-adapted pathogen *S. pneumoniae* in response to Sias, in particular Neu5Ac and Neu5Gc, which are mainly abundant in humans and other mammals, respectively. Despite focusing on infections with pneumococci only, preceding influenza infections, which are known to lead to severe complications, were also taken into account. Moreover, the activation of host immune responses by pneumococcal glycan structures was investigated.

2.1 SPECIFIC AIMS

Paper I

To study pneumococcal pathogenesis and the expression of virulence factors in response to the Sias Neu5Ac and Neu5Gc, as well as to determine how these two sugars effect intracellular signalling in pneumococci.

Paper II

To analyse pneumococcal interactions with Neu5Ac or Neu5Gc on host cells and to characterise the effect of pneumococcal toxins on the evoked host response *in vitro* and *in vivo*.

Paper III

To explore alterations of the host immune response in the lower respiratory tract after influenza infection and their consequences on coinfection with *S. pneumoniae*, as well as to investigate pneumococcal adaptations to this environment.

Paper IV

To identify pneumococcal structures that activate immune responses by dendritic cells and to find a possible mechanism for the enhanced cytokine secretion, observed in influenza/pneumococcal coinfections.

3 METHODOLOGICAL CONSIDERATIONS

This chapter gives a general overview of infectious agents and methods that have been used for the work of this thesis. For a more detailed description of the protocols, see Material and Methods sections of the respective papers.

3.1 BACTERIAL AND VIRAL STRAINS AND THEIR GROWTH CONDITIONS

S. pneumoniae was cultured in C medium with yeast extract (C+Y) [277]. In some experimental set-ups, monosaccharide-free C+Y was supplemented with either 12 mM glucose, Neu5Ac or Neu5Gc. Liquid cultures or bacteria on plates were grown at 37°C in a water bath or at 37°C and 5% CO_2 in an incubator, respectively.

Throughout all the studies in this thesis, encapsulated and invasive pneumococcal serotype type 4, TIGR4 (ATCC BAA-334) [188] or its non-encapsulated derivative T4R (paper II and IV) [278] were used. *In vitro* challenges with T4R were shown to lead to similar numbers of phagocytosed bacteria and to comparable amounts of cytokines produced by the cells compared to studies using an opsonized TIGR4 [266].

Bacterial mutants were constructed by overlap polymerase chain reaction (PCR) of the upstream region, an erythromycin cassette and the downstream region of the target gene [279]. The PCR product containing these three DNA-fragments was then transformed into *S. pneumoniae* TIGR4 or T4R, which was pre-incubated with the competence-stimulating peptide CSP-2 in order to induce competence. All mutations were confirmed by PCR and sequencing.

Since coinfections of IAV and pneumococci are a major cause of mortality, paper III and IV studied different aspects of preceding influenza infections followed by pneumococcal challenges. In paper III, we used the mouse-adapted influenza virus strain PR8/A/34, while in paper IV we used the human-adapted X31. Both were propagated on Madin-Darby canine kidney (MDCK) cells.

3.2 CHARACTERIZATION OF PNEUMOCOCCAL PHENOTYPES

3.2.1 Sialidase activity

Pneumococcal sialidases are major virulence factors [209, 210]. In order to measure sialidase activity of bacterial lysates in paper I, a protocol by Manco *et al.* was used [209]. 2-O-(p-nitrophenyl)-D-*N*-acetylneuraminic acid was added to the samples, followed by an incubation at 37°C. Sialidases are able to cleave this substrate into Neu5Ac acid and p-nitrophenol, which has a yellow color. Thus, the absorbance at 405 nm was measured. The values were compared to a standard curve with known concentrations of the sialidase of *Arthrobacter ureafaciens* and normalized to total protein content in order to determine sialidase activity of the sample.

3.2.2 Production of hydrogen peroxide and acetyl phosphate

The pyruvate oxidase (SpxB) is a pneumococcal virulence factors and responsible for the production of H_2O_2 [231]. To determine amounts of H_2O_2 in samples of paper I and III, we used a previously described method [229]. 2,2´-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) and horseradish peroxidase were added onto the sample. After incubation at room temperature, the absorbance at 560 nm was measured. Total amounts of H_2O_2 were calculated with the help of a standard curve, which was produced with known concentrations of H_2O_2 and normalized to bacterial numbers in the samples.

SpxB was reported to affect intracellular acetyl phosphate (AcPh) levels [231]. In paper I, AcPh content inside the bacterial cell was studied according to the protocol by Pruss and Wolfe [280]. In order to measure total AcPh amounts, it was used to convert adenosine diphosphate (ADP) to adenosine triphosphate (ATP) by an acetate kinase, and quantified using a bioluminescence kit. Finally, the results were compared to a standard curve with known AcPh amounts and normalized to total protein in the sample.

3.2.3 Survival in response to hydrogen peroxide

Although pneumococci have been shown to be catalase-negative, they developed mechanisms to resist ROS [84, 234]. To study the sensitivity of pneumococci to H_2O_2 in paper I and III, the method published by Ibrahim *et al*. was used [220]. Bacteria were incubated in the presence of H_2O_2 at 37°C. The percentage of surviving bacteria was calculated by dividing viable bacterial numbers after exposure by viable bacterial numbers before exposure to H_2O_2.

3.3 IN VITRO AND EX VIVO MODELS

3.3.1 Cells

Since *S. pneumoniae* is a human-adapted pathogen [281], we performed most of the *in vitro* experiments with human cell lines or compared cells with either Neu5Ac- or Neu5Gc-containing glycoconjugates.

In paper II, human cell lines THP-1 (ATCC TIB-202) and A549 (ATCC CCL-185) derived from acute monocytic leukemia or epithelial lung cell carcinoma, respectively, were used. To differentiate THP-1 cells into macrophage-like cells, they were incubated with phorbol myristate acetate. Cell lines present a good *in vitro* model in order to mimic the *in vivo* setting. However, the immortalization of the cells can also lead to differences in their phenotype, e.g. receptor expression levels. Therefore, we additionally performed experiments with primary alveolar macrophages, which were isolated from the BAL from uninfected C57BL/6 wild-type (wt) or *Cmah*$^{-/-}$ mice. While wt mice mainly present Neu5Gc on the surface of their cells, *Cmah*$^{-/-}$ mice have a human like deletion in exon 6 of the *Cmah* gene [282], leading to the lack of Neu5Gc.

In paper IV, primary monocytes from buffy coats of healthy human donors were isolated and cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 to differentiate them into DCs. Successful differentiation was confirmed by the presentation of the surface markers CD1a and CD11c.

Human embryonic kidney 293 cells (HEK293, ATCC CRL-1573) were used in paper IV. These cells were stably transfected with plasmids either expressing TLR3 or TLR4 and luciferase.

3.3.2 Sialic acid feeding of cells

The Sia profiles differ greatly between humans and most other mammals [133, 134]. To study the effect of Neu5Ac- and Neu5Gc-presentation by host cells on pneumococcal virulence in paper II, a previously published method was used [283, 284]. We supplemented the RPMI 1640 cell medium with Neu5Ac and heat-inactivated human AB serum or Neu5Gc and heat-inactivated fetal bovine serum. The Neu5Ac- and Neu5Gc-presention by the cells was confirmed by staining with *Sambucus nigra* lectin (SNA), *Maackia amurensis* lectin II (MAA II) and an antibody raised against Neu5Gc.

3.3.3 Measurement of cytokine response and cell toxicity

In all papers, pro-inflammatory cytokines, like human or murine TNFα or interleukin 6 (IL-6), IL-12, and chemokines, like human IL-8 or murine KC or macrophage inflammatory protein 2 (MIP-2), were determined in cell supernatants or BAL of mice using enzyme-linked immunosorbent assay (ELISA).

3.4 IN VIVO MODELS

Pneumococci have humans as their natural host [281] but different animal models, like rats and mice, have been described as a suitable model to study pneumococcal infections [285]. *In vivo* models allow studying pneumococcal-host interactions in a complex environment, which also takes the immune system as a whole into account. In paper I, II and III, wt and/or *Cmah*$^{-/-}$ mice have been used. Besides, diminishing the synthesis of Neu5Gc, an inactivation of *Cmah* in the mice caused age-related hearing loss and defects in wound healing without affecting the recruitment of immune cells [282]. Similar to humans, *Cmah*$^{-/-}$ mice are also demonstrated to exhibit an over-reacting immune system [143, 144]. A recently reported *dock2* mutation in some *Cmah*$^{-/-}$ mice, which occurred due to backcrossing into commercially available C57BL/6 mice [286], was not present in the mice used in this thesis.

In paper I and II, mice were infected intransally with 20 µl PBS containing *S. pneumoniae*, presenting the natural route of infections by pneumococci. About half of the inoculum reached the lungs directly during infection, allowing us to study the development of pneumonia and the progress into septicaemia in the mice. In paper I, we also infected the mice intravenously, in order to study pneumococcal infections in the blood stream. In paper III, we challenged the mice intranasally with influenza. To study pneumococcal/influenza coinfections, pneumococci were instilled intratracheally, which allowed us to deliver the whole bacterial inoculum into the lower respiratory tract.

3.5 ETHICAL CONSIDERATIONS

All experiments were performed in accordance with the local ethical committee (Stockholms Norra djurförsöksetiska nämnd). Mice were kept with food and water *ad libitum* and 12 hours light/dark cycles. The health status of the mice was controlled regularly.

4 RESULTS AND DISCUSSION

4.1 PAPER I

***Streptococcus pneumoniae* senses a human-like sialic acid profile via the response regulator CiaR**

When pulmonary pathogens, like *S. pneumoniae*, enter the respiratory tract, they come in contact with Sias in mucins [166, 287]. Pneumococci are adapted to humans as their natural host [281]. Thus, the first study included in this thesis determined pneumococcal virulence in response to the Sias Neu5Ac and Neu5Gc, which are predominantly found in mucins of humans or of most other mammals, respectively. Moreover, we compared expression levels of pneumococcal virulence factors in response to these sugars and elucidated how Sia catabolism affects intracellular signalling in pneumococci.

To study pneumococcal pathogenesis in response to Neu5Ac and Neu5Gc, we challenged C57BL/6 wt and *Cmah*$^{-/-}$ mice with pneumococci. While wt mice are able to convert Neu5Ac to Neu5Gc, *Cmah*$^{-/-}$ mice, like humans, overproduce Neu5Ac and are not able to synthetize Neu5Gc [282, 288]. We chose an *in vivo* model of intranasal challenge, since it presents the natural route of infection by pneumococci. At 24 hours post infections, we detected significantly higher bacterial numbers in lungs, BAL and the blood as well as reduced survival rates of *Cmah*$^{-/-}$ compared with wt mice. Hence, pneumococci trigger stronger symptoms of disease in *Cmah*$^{-/-}$ mice in comparison to wt mice. In agreement with our results Trappetti and colleagues observed higher bacterial counts in the lungs of mice after intranasal administration of Neu5Ac but not Neu5Gc [170].

Besides being able to utilize Neu5Ac as carbon source [172, 197], our data indicate that *S. pneumoniae* can also grow on Neu5Gc. Since pneumococci are human-adapted pathogens, we propose that pneumococcal virulence factors mediate increased virulence in response to human-like Sia profiles compared to Neu5Gc. We decided to study the activity of sialidases, since pneumococcal sialidase NanA was shown to bind to Sia and to remove it from the glycan strand [178]. Moreover, sialidases of other bacteria, viruses and mammals have been reported to preferentially adhere to and cleave Neu5Ac- compared with Neu5Gc-containing substrates [289-291]. To catabolize Sia, it needs to be taken up into the bacterial cell [172] and both NanA and the main Sia transporter SatABC have recently been demonstrated to be upregulated in response to Neu5Ac [189, 200]. We isolated pneumococcal RNA from *in vitro* conditions using monosaccharide-free C+Y medium supplemented with either Neu5Ac or Neu5Gc, and *in vivo* from the nasopharynx of wt and *Cmah*$^{-/-}$ mice. *NanA* transcription, as well as sialidase activity was increased in Neu5Ac-incubated bacteria compared to Neu5Gc-treated ones. Moreover, bacteria isolated from the nasopharyngeal lavage of *Cmah*$^{-/-}$ mice had higher *nanA* mRNA levels in comparison to those isolated from wt mice. Additionally, the substrate binding protein SatA of the main Sia transporter was also higher transcribed in

response to Neu5Ac as compared with Neu5Gc. The differential transcription of *nanA* in TIGR4 in response to the two sugars was dependent on the three known Sia transporters, SatABC, SP1688-90, and SP1328 [184, 187], which suggest that pneumococci upregulate virulence factors in response to Neu5Ac compared with Neu5Gc after it has been taken up into the bacterial cell.

To test whether pneumococcal TCS are involved in the Sia-mediated signalling, we monitored transcription rates of all known pneumococcal RR, as well as growth rates of their deletion mutants on Sia and glucose. Our data reveal that a mutant deficient in CiaR had severe growth defects on Neu5Ac and Neu5Gc but not on glucose. Moreover, mRNA expression of RR05, also known as CiaR, was increased in bacteria treated with Neu5Ac compared with Neu5Gc but only if the Sia transporters were present. Hence, we hypothesized that this RR could be involved in intracellular signalling in response to Sia or its metabolic compounds. The TCS CiaRH is known to control the expression of HtrA, which mediates pneumococcal resistance to oxidative stress [234]. Consequently, we measured *htrA* transcription and pneumococcal survival in response to H_2O_2 and found both to be increased after incubation in Neu5Ac compared with Neu5Gc.
CiaR needs to be phosphorylated in order to bind to target promoter sequences [228]. Interestingly, it was shown that its phosphorylation does not depend on the HK CiaH, instead intracellular AcPh can also serve as phosphor-donor [230]. In pneumococci, the pyruvate oxidase SpxB is the main producer of AcPh and H_2O_2 [229, 231]. Moreover, its expression was shown to affect CiaR transcription [230]. After treatment with Neu5Ac, we observed higher *spxB* transcription as well as increased H_2O_2 and AcPh amounts inside pneumococci in comparison to Neu5Gc treatment. Noteworthy, an *spxB*-deficient mutant did not only downregulate *ciaR* transcription, it also decreased *nanA* levels significantly.

CiaR, NanA as well as SatABC are well-known pneumococcal virulence factors [172, 209, 210, 215, 292]. While intranasal infections with wt TIGR4 resulted in higher bacterial numbers in the lungs and blood of *Cmah*$^{-/-}$ compared with wt mice, challenges with TIGR4Δ*ciaR*, TIGR4Δ*nanA* or TIGR4Δ*satABC* led to comparable cfu in the lungs of wt and *Cmah*$^{-/-}$ mice without invading into the bloodstream. Thus, CiaR, NanA and SatABC contribute to a more severe outcome of pneumococcal disease in *Cmah*$^{-/-}$ compared with wt mice.

In TIGR4, the genes required for Sia retrieval and metabolism are encoded in the *nanAB* and the *nanC* loci [178, 184]. The *nanAB* locus harbours catabolite repression elements [189, 200], which suppress the transcription of genes inside the locus in the presence of glucose. Also, CiaR is reported to be downregulated in response to glucose [293]. Since we did not observe any differences in bacterial numbers and survival rates between wt and *Cmah*$^{-/-}$ mice after intravenous challenge, we propose that NanA, SatABC and CiaR facilitate a faster disease progression in the glucose-free respiratory tract [294] of *Cmah*$^{-/-}$ compared with wt mice, but not in the blood stream with high glucose concentrations [295].

This study demonstrates a possible mechanism for increased pneumococcal virulence in response to Neu5Ac in contrast to Neu5Gc. Our data indicate that pneumococci are able to signal in response to Sias via the RR CiaR. We propose a model, in which the presence of Neu5Ac increases expression of sialidase NanA and Sia transporter SatABC, leading to increased scavenging of Sias and higher amounts of Sia or its metabolic products, e.g. pyruvate, inside the bacterial cell. Subsequently, SpxB expression is upregulated, leading to enhanced levels of AcPh and H_2O_2. Increased AcPh amounts inside the bacterial cell upregulate CiaR, which mediates increased resistance to ROS via HtrA and upregulates NanA expression.

4.2 PAPER II

Human sialic acid profiles mediate increased pneumococcal adhesion and immune evasion

The second study of this thesis further characterised pneumococcal host tropism. Since we observed an upregulation of pneumococcal virulence factors in response to Neu5Ac as compared with Neu5Gc in paper I, we aimed at investigating how pneumococci interact with Neu5Ac- or Neu5Gc-presenting host cells and how this interplay affects the host's immune response.

Since it was shown that virulence factors of several human-adapted pathogens favourably bind to human-like sialic acid profiles [140, 142], we set up an *in vitro* model to study pneumococcal interactions with cells presenting either Neu5Ac or Neu5Gc. Sia feeding has been reported to modify glycan structures of several cell lines [283, 284]. We used macrophage-like THP-1 cells and a lung epithelial cell line (A549). By supplementing the cell culture media with Neu5Ac and human serum or Neu5Gc and fetal calf serum, we could detect α2-3- and α2-6-linked Sias by staining the cells with the lectins MAAII and SNA, respectively. However, only after Neu5Gc supplementation could Neu5Gc be detected on the surface of both cell lines using anti-Neu5Gc antibodies. In contrast, cells that were cultured in the presence of Neu5Ac were not recognized by the anti-Neu5Gc antibody. We propose that they most likely present Neu5Ac, although this remains to be experimentally confirmed.

Next, we studied whether Neu5Ac- or Neu5Gc-supplementation of the growth media affects pneumococcal adhesion to cells. We used a non-encapsulated variant of TIGR4, T4R, since it was shown to behave similar to opsonized wt TIGR4 in *in vitro* phagocytosis assays [266]. By performing cell adhesion and uptake assays, we detected higher numbers of cell-associated bacteria when cells were cultured in the presence of Neu5Ac as compared with Neu5Gc. However, we excluded the pneumococcal capsule as determinant of this phenotype, as both wt TIGR4 and T4R adhered better to Neu5Ac- than to Neu5Gc-incubated cells. To test whether increased pneumococcal adhesion to Neu5Ac as compared with Neu5Gc-presenting cells, can be extrapolated to primary cells, we isolated primary alveolar macrophages from uninfected wt and *Cmah*$^{-/-}$ mice. After challenging the cells with pneumococci, we observed higher adhesion rates to macrophages from *Cmah*$^{-/-}$ compared with those from wt mice.

The pneumococcal pyruvate oxidase SpxB and pneumolysin are potent virulence factors and stimulate inflammatory processes [236]. In line with observations of other groups, we detected lower adhesion rates of *spxB* and pneumolysin deficient mutants compared with wt bacteria [263, 296]. Since inflammatory processes are reported to alter the glycan structures on host cells [164, 165], we proposed that these pneumococcal cytotoxins could upregulate glycosylations on cells and hence provide additional attachment sites for bacteria. Moreover, mutants in *spxB* produce significantly lower AcPh amounts than wt bacteria [297]. AcPh is

shown to be able to phosphorylate RR CiaR, a major pneumococcal regulator [230], thus expression levels of adhesins might also be reduced. It will be interesting to test which pneumococcal adhesins favourably bind to Neu5Ac than to Neu5Gc.

More bacteria also lead to higher levels of pneumococcal virulence factors, like SpxB or pneumolysin. Recently, it was shown that the presence of SpxB and pneumolysin cause host cell apoptosis [210, 237, 250, 297, 298]. Consequently, the increased bacterial burden on Neu5Ac-incubated cells might cause higher cytotoxicity in comparison to lower bacterial numbers associated with Neu5Gc-fed cells.

Like T4R, mutants in *spxB* and pneumolysin adhered better to Neu5Ac- than to Neu5Gc-cultured cells. T4R and T4RΔ*spxB* were also taken up more into Neu5Ac- compared to Neu5Gc-treated cells. In contrast, there was no significant difference in phagocytosis of T4RΔ*ply* between Neu5Ac- and Neu5Gc-fed cells. Since pneumolysin has been demonstrated to bind to sialylated carbohydrates on cells [254], we speculate that extracellular pneumolysin facilitates higher pneumococcal adhesion to Neu5Ac as compared with Neu5Gc on host cells.

To study the host's immune response, we measured the production of IL-8 after pneumococcal infection of Neu5Ac or Neu5Gc-incubated THP-1 cells. IL-8 concentrations were reduced in cells fed with Neu5Ac compared with Neu5Gc after challenge with T4R and T4RΔ*spxB* but not after infection with T4RΔ*ply*. It is tempting to speculate that lower IL-8 levels are caused by higher cytotoxicity in Neu5Ac-fed cells, but this remains to be tested.

To determine the importance of Neu5Ac- and Neu5Gc-presenting cells in pneumococcal infections *in vivo*, we challenged wt and *Cmah*$^{-/-}$ mice intranasally with TIGR4, TIGR4Δ*spxB* or TIGR4Δ*ply*. We observed similar bacterial numbers in BAL of wt and *Cmah*$^{-/-}$ mice after challenge with TIGR4 or its isogenic mutants in *spxB* or pneumolysin. However, we detected lower total cell numbers, fewer neutrophils as well as lower MIP-2 and TNFα concentrations in BAL of *Cmah*$^{-/-}$ compared with wt mice after challenge with TIGR4 but not after infection with TIGR4Δ*spxB* or TIGR4Δ*ply*. KC concentrations were lower in *Cmah*$^{-/-}$ than in wt mice after infection with TIGR4 and TIGR4Δ*spxB*. Infections with TIGR4Δ*spxB* or TIGR4Δ*ply* almost did not cause any neutrophil influx in the BAL. To exclude an immunodeficiency of *Cmah*$^{-/-}$ mice, we administered LPS intranasally into wt and *Cmah*$^{-/-}$ mice. Both mice showed a similar immune response and recruited comparable numbers of cells into the airway.

In summary, we observed higher pneumococcal adhesion rates to cells expressing human-like Sia profiles. Moreover, higher bacterial numbers resulted in lower IL-8 secretion, which was mainly mediated by the pneumococcal cytotoxin pneumolysin. A recently published study reported that also *E. coli* adheres better to cells of *Cmah*$^{-/-}$ than wt mice. The authors observed increased bacterial killing after infecting macrophages of *Cmah*$^{-/-}$ mice with sublethal doses of *E.coli* but a higher susceptibility to endotoxins in the knock-out mice after intraperitoneal challenge. They hypothesized that the CMAH loss provided

an advantage to clear minor infections, while it can be harmful during severe infections [135].

In line with our *in vitro* data, we observed lower cytokine and chemokine concentrations as well as lower numbers of infiltrating neutrophils in the BAL of *Cmah*$^{-/-}$ than in wt mice after challenge with TIGR4. Thus, we propose that higher pneumococcal adhesion rates to alveolar epithelial cells and/or macrophages of *Cmah*$^{-/-}$ mice compared with wt mice mediate increased cytotoxicity in the knockout mice. Macrophages and epithelial cells produce cytokines and chemokines, which evoke an influx of neutrophils to the site of infection. Hence, higher cell death rates in these cells might lead to lower secretion rates of inflammatory mediators and lower numbers of recruited neutrophils. However, further experiments are required to test this hypothesis.

Additionally, our data indicate that using cell culture medium supplemented with fetal bovine serum in *in vitro* assays might skew the experimental outcome for human-adapted pathogens, as they might preferentially bind to Neu5Ac and not to Neu5Gc.

4.3 PAPER III

Mechanism for enhanced bacterial burden in the lower respiratory tract of mice during influenza/pneumococcal coinfection

The third study of this thesis determined influenza-mediated alterations in the respiratory tract and their consequences for pneumococcal adaptation and virulence regulation.

To set up an *in vivo* model for studying influenza/pneumococcal coinfections, we challenged mice with pneumococci 7 days post influenza infection. At this time point, the mice were recovering from the primary influenza infection. Additionally, humans are also shown to be highly susceptible to bacterial superinfections about one week post influenza infection [299]. In line with earlier publications [274, 300], we detected higher bacterial burden in the BAL of influenza/pneumococcal coinfected mice compared to those, which were challenged with pneumococci only.

Viral sialidases are known to cleave terminal Sias from the glycan strand [301], hence they increase the concentration of free Sia in the respiratory tract. Siegel and colleagues showed that pneumococci utilize virus-released Sias in the nasopharynx, which promotes pneumococcal colonization and translocation into the lungs [171]. To test whether free Sias also foster pneumococcal dissemination in the lower respiratory tract (LRT), we infected mice with TIGR4 and its isogenic mutant in the main Sia transporter SatABC. Like wt TIGR4, infections with a *satABC*-deficient mutant led to increased bacterial numbers in the BAL and lungs of coinfected compared with single-infected mice. Hence, pneumococcal outgrowth in the LRT is independent on growth on Sia.

To determine total numbers of immune cells and to investigate their contribution to the host response in the LRT, we isolated cells from BAL of untreated, influenza infected, pneumococcal challenged and coinfected mice. As it was reported by other groups [273], the number of macrophages was decreased in influenza and coinfected mice, which might contribute to a defect in bacterial clearance [273, 274, 302, 303]. In contrast, cytokine release and neutrophil numbers were increased after challenge with influenza and in coinfected mice. Thus, although bacterial clearance might be reduced, recruitment of neutrophils and cytokine secretion mechanisms seem to be functional in our model.

AMPs are known to play an importance role in the innate immune response to invading pathogens. To measure the production of AMPs and collectins in alveolar macrophages, neutrophils and epithelial cells, we isolated RNA from lung tissue and transcription of cramp, BD1, BD3, SP-A and SP-D. At day 7 post influenza challenge, transcription of *cramp*, *bd1* and *sp-A* was significantly reduced in comparison to uninfected mice. In coinfected mice, mRNA levels of *cramp* and *bd1* were strongly decreased. These findings are coherent with another study, which reported a downregulation of antimicrobial proteins and peptides in coinfected versus single-infected lungs [304]. Interestingly, we found increased protein

expression of cramp in influenza and coinfected mice compared to uninfected and single-infected ones. On one hand, increased cell death in the respiratory tract can lead to lower production levels of antimicrobial peptides and proteins. On the other hand, recruited neutrophils already contain cramp and release it, thus leading to a negative feedback, which inhibits *cramp* transcription and helps to prevent host cell damage [305].

It is well-known that influenza infections lead to increased oxidative stress in the LRT [306]. To study the effect of influenza-mediated ROS production by immune cells on pneumococcal virulence, we determined concentrations of H_2O_2 in BAL of uninfected and influenza challenged mice. After infection with influenza, we detected higher H_2O_2 levels in BAL than in uninfected mice. Since pneumococcal resistance to ROS is regulated by the serine protease HtrA [234], we measured *htrA* transcription and found it to be increased in response to influenza-infected BAL compared to uninfected BAL. Hence we suggest that influenza leads to enhanced stress in form of denatured proteins and increased ROS in the LRT, which upregulates pneumococcal HtrA. Furthermore, in paper I we found a Sia-mediated upregulation of HtrA. Influenza infections increase the level of free Sia in the airway of infected mice [171], and thus could also mediate HtrA upregulation in pneumococci incubated in BAL of influenza-infected animals.

Genes involved in the regulation of oxidative stress have been shown to be important during coinfections of influenza and *H. influenzae* [307]. However, we did not find any study, which examined the role of HtrA in influenza/pneumococcal coinfections. The serine protease HtrA is surface exposed [241] and a well-known pneumococcal virulence factor [248]. It is demonstrated to have chaperone and protease functions, as it aids protein folding at lower temperatures, and degrades misfolded proteins under stress conditions [242]. Moreover, it has been demonstrated to degrade E-cadherin, thus fostering bacterial invasion across epithelial barriers [246, 247]. We detected higher protein concentrations and increased numbers of dead cells in the LRT of influenza infected mice at day 7 post infections. Thus, we hypothesized that pneumococci might metabolize and grow on denatured proteins and cell debris. To test the role of HtrA in a coinfection setting, we infected mock and influenza-challenged mice with TIGR4Δ*htrA* and observed severely reduced bacterial numbers in the LRT of coinfected in contrast to single-infected mice.

The TCS CiaRH is reported to control transcription of *htrA in vitro* [218, 219]. In contrast to infections with TIGR4Δ*htrA*, infections with a *ciaR*-deficient mutant led to similar bacterial numbers as challenges with wt TIGR4. Moreover, our results suggest that HtrA is required to cope with stress in the LRT post influenza challenge and that *htrA* transcription can be controlled independently of CiaR in the lungs of coinfected mice. Interestingly, RR09 has been implicated in pneumococcal virulence, as well as in regulation of HtrA expression [308]. Future studies will show, if RR09 is responsible for the upregulation of HtrA *in vivo* in the absence of CiaR.

In summary, our data suggest that influenza-mediated changes of the host environment in the LRT support secondary pneumococcal infections by promoting pneumococcal adaptation and

upregulating pneumococcal virulence factors, like HtrA. Moreover, HtrA facilitates pneumococcal outgrowth during influenza/pneumococcal coinfections, which indicates its importance as a therapeutic target.

4.4 PAPER IV

Toll-like receptor 3/TRIF-dependent IL-12p70 secretion mediated by *Streptococcus pneumoniae* RNA and its priming by influenza A virus coinfection in human dendritic cells

The last study of this thesis describes the recognition of pneumococcal RNA by TLR3 and presents a mechanism for the increased IL-12 production in DCs upon influenza/pneumococcal coinfection.

DCs are shown to be the main producers of IL-12p70. After pneumococcal infections, IL-12 activates T_H1-cells and stimulates the production of IFN-γ by other immune cells [115-117]. IL-12 plays an important role *in vivo*, as its exogenous administration improved the disease outcome in a murine model of pneumococcal pneumonia [309]. Moreover, IL-12 deficiency in humans was associated with recurrent pneumococcal infections [310]. To examine the role of this cytokine in pneumococcal infections in monocyte-derived DCs, we challenged these cells with a low infection dose of T4R. A MOI of 1 did not affect cytotoxicity but evoked a secretion of IL-12p70 by DCs, which was dependent on pneumococcal uptake.

Next, we aimed at studying the role of TRIF in pneumococcal infections of DCs. TLR3 and TLR4 are the only PRRs that signal via TRIF, while other TLRs use MyD88 as adapter molecule to stimulate host responses [61, 62]. We silenced TRIF in DCs using small interfering RNAs and challenged the cells with T4R to measure IL-12p70 production. RNA as well as protein levels of IL-12 were decreased. Hence, DCs sense pneumococci via TRIF-mediated TLR-signalling.

To determine the role of TLR4 in pneumococcal sensing by DCs, we next silenced TLR4 in these cells and measured IL-12p70 secretion. While there are studies implicating pneumococcal toxin pneumolysin in the activation of TLR4 [67, 311-313], other research groups have observed a TLR4-independent sensing of pneumolysin [71, 251, 314]. Our data indicate that pneumococci can activate DCs independently of TLR4 expression in this model, as IL-12 transcription and secretion were not affected by inactivation of this PRR.

Next, we silenced TLR3 in DCs and measured IL-12p70 production after pneumococcal challenge. Interestingly, we found a decrease of this cytokine on transcriptional as well as on protein level. To confirm this result, we made use of a TLR3/dsRNA complex inhibitor, which reduced the secretion of IL-12. The capsule did not interfere with this result, as we observed a downregulation of IL-12 release even after challenge with wt TIGR4. These results show that DCs recognize pneumococci via the endosomal receptor TLR3.

A previous study by our group reported an IAV-mediated type I IFN production, which led to an enhanced secretion of IL-12 by neighbouring DCs [315]. Moreover, viral infections as well as type I IFN are known to upregulate TLR3 expression [316, 317]. To explore whether IAV upregulates TLR3 and consequently leads to an increase of IL-12p70 secretion in

influenza/pneumococcal coinfections, we measured mRNA levels of TLR3 in DCs challenged with influenza alone and after influenza/pneumococcal coinfection. Indeed, TLR3 expression was enhanced in IAV infected and coinfected DCs. Moreover, we tested IL-12p40 transcription and found it to be upregulated in T4R single-infected and even more in IAV/T4R coinfected DCs. However, treatment with IFN-α alone only had a minor effect on TLR3 expression, suggesting that additional factors other than IFN-α might contribute to the influenza-induced TLR3 upregulation.

TLR3 is known to recognize viral dsRNA [62]. However, there are recent reports describing TLR3-mediated sensing of bacterial RNA [318-320]. To test whether pneumococcal RNA is sufficient to induce TLR3-mediated IL-12p70 production, we isolated RNA from T4R and transfected it into DCs. We observed an induction of IL-12p70 secretion upon transfection with total RNA, which was dependent on TLR3 and completely abolished after RNAse-treatment. Furthermore, we confirmed RNA-mediated TLR3 activation in HEK293 cells expressing TLR3.

Additionally, we killed pneumococci by heat exposure and UV radiation and found the latter condition to induce IL-12p70 secretion. We determined the RNA quality after the two treatments and found the RNA to be degraded after heat treatment, while UV radiation maintained RNA integrity. Moreover, our data show that RNAse treatment of UV-killed pneumococci reduced cytokine secretion in a dose-dependent manner, while DNAse treatment and LPS transfection in combination with RNAse did not affect IL-12 production.

In summary our data indicate that pneumococci are taken up into DCs and degraded inside the endosome. Released pneumococcal RNA can subsequently be sensed by TLR3 and induces secretion of IL12-p70. Furthermore, our results give a possible explanation for the increased cytokine secretion in influenza/pneumococcal coinfection. The data suggest that infections with influenza lead to the secretion of type I IFN and other factors, which enhances TLR3 expression by DCs. Consequently, the higher level of TLR3 mediate increased IL12-p70 production in response to secondary pneumococcal infections.

5 CONCLUDING REMARKS

S. pneumoniae is a major threat to human health, especially of very young, elderly and immunocompromised individuals. Even though vaccines and antibiotics are available today, the emergence of non-vaccine types in IPD and antibiotic resistance in pneumococci demand for the development of improved vaccines and therapeutics. Consequently, a comprehensive understanding of pneumococcal-host interactions is required.

Immediately after entering the host, pulmonary pathogens, like *S. pneumoniae* or IAV, come in contact with glycans, which serve as receptors for adhesion [166] and thus present a prerequisite for pathogenesis. In the case of pneumococci, sugars of the glycan strand can also act as carbon source [172, 197, 293]. *S. pneumoniae* allocates more than 30% of all transporters to the uptake of carbohydrates [188], which points towards the importance of carbohydrates in pneumococcal colonization and disease. Sialic acid, the terminal sugar of the glycan strand, has been implicated in pneumococcal metabolism but has also been demonstrated to serve as a signal for pneumococcal disease [170, 172]. However, there is also another aspect of glycan-based host-pathogen interactions, as most PAMPs are demonstrated to be microbial glycan structures, and thus activate innate immune responses [181].

In paper I and II, we study sialic acid-mediated aspects of pneumococcal host tropism. In paper I, we demonstrate that pneumococci upregulate several virulence factors in response to Neu5Ac, the sialic acid mainly abundant in humans, compared with Neu5Gc, which cannot be synthesized by humans. Moreover, we show that sialic acid or its metabolic products are sensed involving the pneumococcal response regulator CiaR. In paper II, we show an increased pneumococcal adhesion rate to Neu5Ac– compared with Neu5Gc-containing host glycans. Moreover, we present data indicating a role of pneumococcal cytotoxins in increased immune evasion by pneumococci in response to Neu5Ac compared with Neu5Gc.

In paper III and IV, we study pneumococcal-host interactions after prior influenza infections, since they account for severe disease outcomes. In paper III, we determine influenza-induced changes in the LRT of mice and investigate pneumococcal adaptations to this environment. In paper IV, we show that pneumococcal RNA can be sensed by TLR3 in DCs. Moreover, in a coinfection setting, influenza infections increase TLR3 expression with the help of type I interferon. Thus upon secondary pneumococcal challenge, enhanced TLR3 expression upregulates IL-12 production.

In summary, we show that glycans play a pivotal role in pneumococcal pathogenesis. The studies included in this work give insights into alterations of pneumococcal-host interactions caused by differences in the sialic acid profile of humans in comparison to other mammals, as well as by preceding viral infections. Moreover, this thesis describes the activation of the innate immune system by pneumococcal glycan-based structures, like RNA. These findings will hopefully add to the improvement of current treatment options and to the development of new vaccines.

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